An investigation of cefotaxime resistance in aerobic Gram-negative bacilli isolated from surveillance flora of patients undergoing a selective parenteral and enteral antisepsis regimen in the intensive therapy unit

Katherine M Hadley

BSc(Med Sci) MBChB FRCPath

A thesis submitted to the University of Edinburgh for the degree of Doctor of Medicine (MD) June 2000

Table of Contents

Page

Index of Figures and Tables iv
Acknowledgements v
Declaration vi
Dedication vii
Abbreviations viii
Abstract x
Aims of the Study xii

Chapter 1 Clinical and Scientific Background

1.1 Introduction 1
1.2 The ITU and the importance of infection 5
1.3 Intensive care patients 6
1.4 The importance of AGNB in ITU infections 7
1.5 The conventional approach to ITU infection 8
1.6 The novel approach to ITU infection 8
1.7 The ß-lactam antibiotics 13
1.8 "Penicillin- and cephalosporin-destroying enzymes" and ß-lactamases 20
1.9 Genetics of antibiotic resistance 22
1.10 ß-Lactamases of Gram-negative bacteria from the 1960s onwards 30

Chapter 2 Patients, Materials and Methods

2.1 Isolates and patients 42
2.2 Antimicrobial susceptibility testing 44
2.3 Plasmid analysis 49
2.4 Transconjugation experiments: test and control systems 68
2.5 ß-Lactamase analysis 81
2.6 Additional transconjugation studies on four isolates which were positive in the Etest for ESBL production 90

Chapter 3 Results

3.1 Isolate collection 95
3.2 Antimicrobial susceptibility testing 95
3.3 Plasmid analysis 97

3.4 ß-Lactamase analysis 99
3.5 Transconjugation experiments 100
3.6 Additional transconjugation studies on the two *Klebsiella* isolates and two *Acinetobacter* isolates which were positive in the Etest for ESBL detection 103
3.7 Individual genera 118

Chapter 4 Discussion

4.1 Aims of the study 145
4.2 Plasmid analysis 145
4.3 Transconjugation experiments 146
4.4 Preparation of cell lysates 147
4.5 Isoelectric focusing, antibiogram and prediction of ß-lactamase type 147
4.6 Chromosomal ß-lactamases, their mode of production and contribution to resistance 148
4.7 Individual genera 151

Chapter 5 Conclusions and Recommendations

5.1 Percentage of patients carrying cefotaxime-resistant AGNB 175
5.2 Cefotaxime resistance unlikely to be related to SPEAR 175
5.3 Cefotaxime resistance directly related to SPEAR 175
5.4 Cefotaxime resistance indirectly related to SPEAR 176
5.5 Chromosomal ß-lactamases 176
5.6 ESBLs 176
5.7 Practical recommendations for the future 177

References 179
Appendix I 200
Appendix II 205
Appendix III 208
Appendix IV 213
Appendix V 218

Index of Figures and Tables

Figures: **Page**

Figure 1 14
Figure 2 19
Figure 3 35
Figure 4 43
Figure 5 45
Figure 6 64
Figure 7 69
Figure 8 70
Figure 9 77
Figure 10 86
Figure 11 89
Figure 12 91
Figure 13 92
Figure 14 98
Figure 15 101
Figure 16 102
Figure 17 105
Figure 18 106
Figure 19 109
Figure 20 110
Figure 21 112
Figure 22 113
Figure 23 114
Figure 24 115
Figure 25 116
Figure 26 117

Tables: **Page**

Table 1 79
Table 2 96
Table 3 104
Table 4 108

Acknowledgements

I wish to thank my adviser Professor SGB Amyes, Department of Medical Microbiology, The University of Edinburgh Medical School for his help and support.

I am grateful to Dr SR Alcock, Department of Clinical Microbiology, Western Infirmary, Glasgow for access to the bacterial isolates.

I should like to acknowledge grant funding for isoelectric focusing equipment from the Research Support Group of Greater Glasgow Health Board.

Declaration

I declare that this thesis has been composed entirely by myself, that all of the scientific and technical work has been carried out by me personally and that the thesis has not been submitted for any other degree, diploma or professional qualification.

Katherine M Hadley

Dedication

To my beloved Donald, my husband and best friend.

Abbreviations

General:

6-APA	6-aminopenicillanic acid
7-ACA	7-aminocephalosporanic acid
AGNB	aerobic Gram-negative bacilli
c.	circa (about)
CCC	covalently closed circular
Col	colicinogenic
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ESBL(s)	extended-spectrum ß-lactamase(s)
F	fertility
HMS	Hedges, Matthew, Smith
ITU(s)	intensive therapy unit(s)
kb	kilobase pairs
MDa	megadaltons
MIC	minimum inhibitory concentration
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
OC	open circular
PBP(s)	penicillin-binding protein(s)
PBS	phosphate buffered saline
*p*CMB	*para*-chloromercuribenzoate
pI(s)	isoelectric point(s)
{p "n"}	page "n" of a book reference
R	resistance
RNA	ribonucleic acid
RTF	resistance transfer factor
SDD	selective decontamination of the digestive tract
SDS	sodium dodecyl sulphate
SENIC	The Study on the Efficacy of Nosocomial Infection Control
SPEAR	selective parenteral and enteral antisepsis regimen
TBE	"tris-borate-EDTA" (tris[hydroxymethyl]aminomethane-borate ethylenediaminetetraacetic acid)

Antibiotics:

AMC 30	"co-amoxyclav" (amoxycillin 20 µg and clavulanic acid 10 µg)
AMP 10	ampicillin 10 µg
ATM 30	aztreonam 30 µg
AZL 75	azlocillin 75 µg
CAR 100	carbenicillin 100 µg
CAZ 30	ceftazidime 30µg
CIP 5	ciprofloxacin 5 µg
CL 30	cephalexin 30 µg
CN 10	gentamicin 10 µg
CTX 30	cefotaxime 30 µg
E 15	erythromycin 15 µg
ESBL_TZ	MIC of ceftazidime 0.5-32 mg/L
ESBL_TZL	MIC of ceftazidime 0.064-4 mg/L with clavulanic acid 4 mg/L
FOX 30	cefoxitin 30 µg
IPM 10	imipenem 10µg
MEL 25	mecillinam 25 µg
MEM 10	meropenem 10 µg
OX 1	oxacillin 1 µg
P 10	penicillin G 10 units
PRL 100	piperacillin 100 µg
TEM 30	temocillin 30 µg
TIC 75	ticarcillin 75 µg
TIM 85	"timentin" (ticarcillin 75 µg and clavulanic acid 10 µg)
TZP 110	"tazocin" (piperacillin 100 µg and tazobactam 10 µg)
W 5	trimethoprim 5 µg

Abstract

This thesis presents a critical evaluation of the clinical and microbiological background to the study of infection in the intensive therapy unit (ITU), the development and importance of the ß-lactam antibiotics and the mechanisms and genetics of resistance to them, with emphasis on the commonest and continually evolving cause of ß-lactam resistance, the ß-lactamases.

A selective parenteral and enteral antisepsis regimen (SPEAR) comprising three components: selective decontamination of the digestive tract (SDD), systemic cefotaxime and intensive microbiological surveillance was applied to patients in a local medical/surgical ITU, with encouraging results. After the formal trial the decision was made to continue with SPEAR. Although clinical problems with antimicrobial resistance were not encountered, this routine use of prophylactic systemic cefotaxime prompted fears of promoting resistance not only to cefotaxime but to the ß-lactam antibiotics in general by:

- the selection in the ß-lactamase-inducible species of aerobic Gram-negative bacilli (AGNB) of stably derepressed mutants which hyperproduce chromosomal AmpC ß-lactamases and
- the selection of plasmid-mediated extended-spectrum ß-lactamases (ESBLs).

Both types of enzymes attack multiple ß-lactam antibiotics.

The objective was to investigate those AGNB from surveillance flora showing resistance to cefotaxime with reference to plasmid-mediation and ß-lactamase characterization using molecular biology techniques.

- One hundred and seventy-five of 200 isolates collected over a period of two years and ten months fulfilled the criteria for the study.
- Plasmids were detected in 78 of 175 isolates by agarose gel electrophoresis with sizes ranging from 2 kb to 214 kb.

- Transconjugation experiments were carried out on all isolates. Both test and control systems were used.
- ß-Lactamases were detected in 144 of 175 isolates by isoelectric focusing.
- All isolates were tested for susceptibility to a battery of ß-lactam antibiotics chosen to facilitate the recognition of the resistance patterns associated with particular ß-lactamase types. Disk diffusion and agar dilution methods were used.
- All isolates were tested for the production of ESBLs. Potential ESBLs were found in four isolates and their relevance is discussed.
- There was evidence of chromosomal ß-lactamase derepression in 65 of 175 isolates.

This is the first such study of AGNB from the surveillance flora of patients exposed simultaneously to cefotaxime and an effective SDD regimen in an ITU.

- Its results were favourable to SPEAR.
- Intensive microbiological surveillance should remain an integral part of that triple regimen.

Aims of the Study

- to investigate the cefotaxime resistance mechanisms of aerobic Gram-negative bacilli isolated from surveillance flora of patients undergoing a selective parenteral and enteral antisepsis regimen (SPEAR) in an intensive therapy unit.

- to assess the implications of such resistance mechanisms for the continuation of SPEAR which comprised selective decontamination of the digestive tract, systemic cefotaxime and intensive microbiological surveillance.

Chapter 1 Clinical and Scientific Background

1.1 Introduction

> ".....so many of our outstanding technical achievements and brilliant examples of dogged devotion to day-to-day care still fail because of our fundamental inability to control the infectious process. For some 30 years we have sought with increasing sophistication, cost, and possible danger, the magic bullet - a new and perfect antimicrobial agent. As each new agent has evolved and been tested, it is ever clearer that the microbial adjustment to the new therapy is so rapid that we have only changed the uniform of our enemy while maintaining a stable battle line." [1]

Infection is one of the most serious problems in the management of intensive care patients. Infection-related morbidity and mortality remain unacceptably high. Once a cycle of infection, reduced host defence and organ failure becomes established conventional management is clearly inadequate and mortality exceeds 50% [2]. Until 1984 the prevention and control of infection in the intensive therapy unit (ITU) was managed by combining detailed clinical and microbiological surveillance, antibiotic policies and isolation procedures. In theory surveillance allowed early detection of infection or pre-infection states and identified the causal organisms, the antibiotic policies restricted drug use thereby minimising selection pressure for resistance and isolation procedures prevented the transmission of infection within the units.

Disenchanted by the legacy of the conventional approach to infection in intensive care patients a Dutch team in Groningen [3] developed a preventative approach based on the observation that many infections are endogenous i.e. caused by potentially pathogenic microorganisms colonizing the patient's own digestive tract. Stressing the importance of endogenously-derived infection by aerobic Gram-negative bacilli (AGNB) and the protective role of the anaerobic intestinal flora in

colonization resistance, Stoutenbeek et al. [3] applied selective decontamination of the digestive tract (SDD), a familiar concept in the management of cancer patients, to the novel setting of the ITU. It should be emphasized that this first application of SDD to patients in the ITU was in fact part of a prophylactic triple regimen which combined three distinct elements: SDD applied throughout the duration of stay in the ITU, systemic administration of the third-generation cephalosporin cefotaxime directed against early endogenous infection and intensive microbiological monitoring throughout the admission.

SDD was achieved with a mixture of tobramycin, polymyxin E and amphotericin B applied as a sticky paste to the oropharynx and as a fluid by nasogastric tube to the stomach and more distal intestinal tract even in the absence of peristalsis. The aim of the SDD regimen was to eliminate or markedly reduce the number of potentially pathogenic AGNB and yeasts in the gastrointestinal tract thereby directly reducing the risk of endogenous infection with these organisms. The highly selective action of the antibiotics used in such SDD regimens allows retention of the predominantly anaerobic flora of the gastrointestinal tract. This retained flora prevents colonization with organisms resistant to the antibiotics used for SDD, a phenomenon termed "colonization resistance" [4,5]. The systemic administration of cefotaxime was directed against early endogenous infection and was discontinued as soon as the microbiological monitoring showed that the patient was free of potentially pathogenic microorganisms [3].

In 1981 Stoutenbeek and colleagues had started a pilot study on infection prevention by SDD, initially without additional systemic antibiotic prophylaxis but

> "As this appeared not optimal, a systemic colonization resistance-indifferent antibiotic such as Cefotaxim was added to the regimen, assuming that the combination with SDD prevents emergence of resistance" [3].

The use of this prophylactic triple regimen achieved a marked reduction in the colonization of the oropharynx and rectum with AGNB and a reduction in the

incidence of unit-acquired infection. Problems with antibiotic resistance were not encountered. In fact environmental studies [6] suggested a beneficial secondary effect on the bacterial ecology of the ITU with a consequent reduction in the risk of exogenous infection with AGNB. Although impressive, the results of these studies applied only to a highly selected group of patients, i.e. 63 multiple trauma patients who were neither infected nor receiving antibiotics at the time of admission and whose duration of stay in the ITU was for five days or longer.

Intrigued by the Dutch findings a Scottish team [7] applied a similar selective parenteral and enteral antisepsis regimen (SPEAR) to all 324 patients admitted to a non-specialized medical/surgical ITU in Glasgow, Scotland over a period of eight months. This prospective trial studied a control group of 161 patients who were managed traditionally followed by a test group of 163 patients managed with SPEAR. As in the Groningen regimen the three components of SPEAR comprised SDD, systemic cefotaxime and intensive microbiological monitoring.

Intravenous cefotaxime was used as a supplement to SDD during the first four days to provide additional broad-spectrum cover for the early period of admission. During this time SDD is only partially established, intubation and other invasive procedures are very common and subclinical infection undiagnosed at admission may become manifest.

The SPEAR group of patients in the Glasgow study showed a striking and consistent reduction in the colonization of the oropharynx, stomach and rectum with AGNB and a statistically significant reduction (24% to 10% $p=0.006$) in the incidence of unit-acquired infection. Mortality in certain categories of patient was also reduced but statistical significance could be reached only in relation to trauma patients. Problems of drug resistance were not encountered [7].

Although the results of the study strongly suggested that SPEAR represented a substantial advance in the control of unit-acquired infection in a general ITU [7], the

widespread application of such a regimen has remained controversial because of concern about the emergence of serious drug resistance [8-12].

In particular the prospect of the routine use of prophylactic cefotaxime (an extended-spectrum ß-lactam) in ITUs prompted fears of promoting resistance not only to cefotaxime itself but to many other ß-lactam antibiotics [8]. Cefotaxime is a third-generation cephalosporin which possesses broad-spectrum high potency antimicrobial activity and stability to a wide range of ß-lactamases [13 {p90}]. Although problems with resistance were not encountered clinically during the Scottish SPEAR trial [7], it is known that selection processes within the normal flora often precede the emergence of clinically important multiply-resistant bacteria [10]. Problems may arise from the complex selection pressures in an ITU which affect not only the individual patient but the patient's total microbiological environment.

After completion of the trial in Glasgow the decision was made to continue SPEAR. Intensive microbiological surveillance remained an integral part of the regimen. This surveillance entailed the routine microbiological examination of tracheal and gastric aspirates, urine, and swabs from the throat and rectum of every patient within four hours of admission to the ITU and three times weekly throughout the patient's stay in the unit in order to monitor resistance. Two hundred consecutive isolates of AGNB from the surveillance flora showing resistance to any component of SPEAR were stored on nutrient agar slopes in darkness at room temperature.

ß-Lactams such as cefotaxime favour the selection of stably derepressed mutants in certain species of AGNB. These mutants produce high levels of chromosomal AmpC ß-lactamases and have reduced susceptibility to multiple ß-lactams [14]. Such multiply resistant organisms have spread widely in some hospitals and this trend has been correlated closely with the extent of use of third-generation cephalosporins [15-18]. Plasmid-mediated extended-spectrum ß-lactamases (ESBLs)

attack not only cefotaxime but most ß-lactams. Their potential for spread among AGNB and dissemination outside the ITU is well known [19-24].

The aim of the present work was to investigate the resistance mechanisms of those AGNB showing resistance to cefotaxime, with reference to plasmid-mediation and ß-lactamase characterization and to assess their implications for SPEAR.

One hundred and seventy-five of the 200 consecutive isolates collected over a period of two years and ten months (5/8/88 to 20/5/91) were appropriate for inclusion in the present study. This is the first such study of AGNB isolated from the surveillance flora of patients exposed simultaneously to systemic cefotaxime and an effective SDD regimen in a general ITU. The results will have important implications for the long-term validation of SPEAR.

1.2 The ITU and the importance of infection

Specialised patient-care units providing a focus for the technological rescue of critically ill patients have been evolving since their introduction in the late 1950s [25] and have become one of the hallmarks of modern medicine. These ITUs are characterized by small groups of severely ill patients with compromised host defences, the routine use of multiple medical devices and treatments with inherent side effects and high ratios of specially trained medical and nursing personnel. They may be subspecialized into neonatal, paediatric, cardiac, respiratory, medical, surgical or neurological or may have mixed facilities such as in a general medical/surgical unit depending on the needs and expertise in the individual hospitals.

Infection has always been a serious problem in the management of intensive care patients. In contrast to general wards with an average nosocomial infection rate of 5-10% the incidence of such infections in ITUs is usually much higher [26]. Awareness of the situation intensified in the middle 1970s through the 1980s [26-30] when an overall incidence of infection of 18%-36% in general medical/surgical

ITUs was reported frequently [28,31,32]. There was a high incidence of unit-acquired infection; the proportion of infected patients increased with duration of stay and could exceed 90% in patients remaining in the unit for longer than seven days [3,28,30].

1.3 Intensive care patients

A patient requiring intensive care may be defined as one who is severely ill and who requires support of one or more vital functions until a disease process is arrested or ameliorated. Patients may be admitted directly from the community or may be transferred from other wards in the same hospital or from other hospitals.

Nowadays it is possible to support seriously ill patients who would previously have died. Patients with major organ failure are kept alive with dialysis, ventilation, transfusions and other sophisticated interventional procedures. Such patients are highly susceptible to infection due in part to compromised host defences which often accompany the underlying disease or trauma. The Study on the Efficacy of Nosocomial Infection Control (SENIC) project [33] confirmed that the risk of nosocomial infection is positively associated with the severity of the underlying illness. Broken skin and mucosal surfaces are immediately colonized as part of the mechanism of injury, allowing microorganisms to bypass otherwise healthy skin and mucosa. Colonization with nosocomial organisms occurs quickly in the intensive care settings necessary for the treatment of patients with multiple trauma and correlates well with the severity of injury and presence of invasive devices [34,35]. Once colonized, wounds, drains and catheters are resistant to microbial clearance by host immune defences. Intensive care patients need various forms of instrumentation to monitor vital functions and support failing organ systems. The epithelial barrier of the skin and mucous membranes is the most important component of normal antibacterial defences. Vascular cannulae mechanically breach skin barriers and provide a direct portal of entry for microorganisms into the bloodstream. Tubes and

catheters inserted into airways and into the gastrointestinal and urinary tracts circumvent local defence mechanisms. Epithelial colonization develops rapidly following admission to an ITU [36]. The critically injured patient loses the protective colonization immunity provided by normal intestinal tract flora as injury, ischaemia, impaired motility, pH changes, fasting and antibiotics disrupt the normal microenvironment. Agents such as antacids and cimetidine (a histamine type 2 blocker or "H2 blocker") given to reduce gastric secretions in the prophylaxis of stress ulcer and haemorrhage alter both the qualitative and quantitative bacterial flora of the stomach. Alterations in this important chemical barrier have implications for both respiratory and gastrointestinal infections. Traumatic injury produces a hypermetabolic state that results in a relative nutritional deficiency, potentially compromising wound healing and host immunity [1].

1.4 The importance of AGNB in ITU infections

In all ITUs septicaemia, pneumonia, infections of the skin and subcutaneous tissue, upper respiratory tract, urinary tract and wound infections are the most common nosocomial infections [26]. However nosocomial infections of the respiratory tract are particularly worrying as they not only complicate and extend hospitalization times but also tend to be lethal [37]. They have been reported to account for 37% to 66% of ITU infections and there are often intractable difficulties in both diagnosis and treatment [30,38,39].

A wide range of microorganisms is associated with ITU infections. However several studies emphasise the importance of AGNB [27,28] especially in unit-acquired infections [30,32,38-43]. The microorganisms include *Escherichia coli*, *Klebsiella*, *Proteus*, *Enterobacter*, *Citrobacter* and *Serratia* species, *Pseudomonas aeruginosa*, *Acinetobacter* species, *Morganella morganii* and *Stenotrophomonas* (*Xanthomonas*) *maltophilia* [32,34,44]. Within this context AGNB is a useful collective term for these organisms.

1.5 The conventional approach to ITU infection

Until the middle 1980s it was generally recommended that antibiotic prophylaxis should not be used routinely in patients in an ITU because apart from being ineffective it might encourage the emergence of resistance in nosocomial AGNB [27,45]. Detailed clinical and microbiological surveillance was used to detect early infection and identify the causal organisms. Systemic antibiotics were given when there was clinical evidence of infection and the spread of resistant organisms was limited by isolation procedures.

1.6 The novel approach to ITU infection

1.6.1 The importance of endogenous infection in the ITU setting

Although the importance of the digestive tract flora as a source of infection had been demonstrated in leukaemic and granulocytopaenic patients [46-48] and also in ITU patients [36,37,49], this aspect had been overshadowed previously in the ITU setting by the importance of exogenous infections [27,41].

In 1983, in a novel approach to infection control in the ITU, Dutch workers [50] focused attention on the importance of endogenous infection. In agreement with other workers [51,52] they recognized that the patient's own aerobic digestive tract flora in the oropharynx, stomach and intestines was the major source of all infections with the other major organ systems such as the respiratory tract, urinary tract and skin being the target organs.

1.6.2 Colonization and infection

Colonization had been defined by Northey and colleagues in 1974 [34] as the appearance of a microorganism in, or on, a patient without clinical signs of disease. They also defined infection as the presence and multiplication of organisms in, or

on, a patient with some clinical signs or symptoms of disease such as an elevated temperature or a wound with a purulent exudate.

The patient's own endogenous flora may change profoundly following admission to a hospital environment and the patient may be colonized by endemic hospital strains of pathogens which may subsequently cause infection. Therefore many apparently endogenous infections are really exogenous in origin [27].

The Dutch team [3] who first applied the concepts of SDD to ITU patients defined colonization as the presence of the same species of potentially pathogenic microorganism in an organ system for more than 3 days (> 2 positive samples) without signs of infection. They defined infection according to the criteria described above [34].

Infection was termed endogenous when it was caused by microorganisms that were part of the patient's oropharyngeal or gastrointestinal flora [53] and they defined the sequence of events in the pathogenesis of endogenous infection as acquisition of potentially pathogenic microorganisms, followed by oral or gastrointestinal colonization which was recognized as a prerequisite for infection by these microorganisms. Primary endogenous infection was distinguished from secondary endogenous infection. According to van Saene [53] and colleagues admission flora is involved in primary endogenous infection, while microorganisms acquired during a hospital stay are associated with secondary endogenous infection. Although the microorganisms involved in secondary endogenous infection are acquired in hospital ("exogenous" microorganisms), the infection is called endogenous because oral and gastrointestinal colonization form an essential stage in the development of infection. An exogenous infection was defined by the Dutch workers as an infection caused by microorganisms not present in the patient's oral and gastrointestinal flora, e.g. lower airway infections associated with ventilation equipment and humidifiers [53]. According to the above criteria ITU-acquired

infection may be either secondary endogenous infection or exogenous infection, but never primary endogenous infection.

1.6.3 Effect of illness on oropharyngeal colonization

A breakthrough in the understanding of the pathogenesis of ITU infections occurred when Johanson reported in his pivotal paper in 1969 [54] that serious illness itself predisposed to oropharyngeal carriage of AGNB. He noted that the prevalence of AGNB among the oropharyngeal bacterial flora was low in physiologically normal subjects despite hospital exposure but rose markedly in patients with illnesses of varying severity. This increased prevalence did not correlate with antibiotic administration or inhalation therapy and did not depend on duration of hospitalization but correlated best with the clinical severity of illness. He contended that increased exposure to these organisms alone did not adequately explain the findings but that pharyngeal clearance mechanisms were impaired in these patients. As most bacterial pneumonias begin with the aspiration of bacteria present in the upper respiratory tract, he concluded that this alteration in the pharyngeal flora of ill patients might represent an important initial step in the pathogenesis of pneumonia due to AGNB. The importance of the progression from abnormal colonization on a surface to overt tissue infection is exemplified by the well documented colonization of the oropharynx, stomach and trachea with AGNB prior to the development of lower respiratory tract infection with these organisms [37,55,56]. Increased oropharyngeal colonization with AGNB is also related to old age and to antibiotic use [57].

1.6.4 Influence of pH on gastric colonization

In critically ill patients a gastric pH >4 has been shown to be the major risk factor for gastric colonization with AGNB [29]. One reason why such patients may have a gastric pH >4 is the use of H2 blockers or antacids as prophylaxis against stress

ulceration and haemorrhage [58]. Driks and colleagues [56] showed a nosocomial pneumonia rate twice as high in an antacid/H2 receptor-antagonist group compared with a group receiving sucralfate which preserves the natural gastric acid barrier against bacterial overgrowth. However critically ill patients may have a gastric pH > 4 even in the absence of antacid or H2 receptor-antagonist therapy [56,59].

1.6.5 Colonization resistance and the role of the indigenous anaerobic flora

In contrast to aerobes, anaerobic bacteria are rarely described as a cause of unit-acquired infection [41] although they may be important in sepsis present on admission [28]. In fact anaerobic bacteria as part of the indigenous flora are thought to play a major role in the host's colonization resistance which can be defined as the host's capacity to prevent colonization and infection with aerobic bacteria. As the digestive tract is the main reservoir of AGNB (the cause of most episodes of ITU infection) its resistance to colonization is of great importance. The principle of colonization resistance, which concerns the barriers that must be overcome before microorganisms can colonize body surfaces, was formulated by van der Waaij [4,5,60] on the basis of findings in mice and monkeys.

In 1971 van der Waaij and colleagues [4] investigated the occurrence of intestinal colonization after oral administration of various doses of three different species of AGNB in mice. Much higher oral doses of bacteria were required to achieve colonization for two weeks or longer in animals with a "conventional" microflora than in those whose microflora had been altered by prolonged oral antibiotic treatment. Van der Waaij and colleagues [4] used the term "colonization resistance" to describe this phenomenon and expressed the term quantitatively as the logarithm of the oral dose of an organism resulting in colonization of the intestinal tracts of 50% of the experimental animals for at least 2 weeks. The concept of colonization resistance was defined by van der Waaij [61] in broader terms as the resistance which a potentially pathogenic microorganism encounters when it tries to colonize a

"landing site" on the mucosa of one of the three tracts (respiratory, alimentary and urinary) that have an open communication with the exterior. In the respiratory and the urinary tracts the colonization resistance determining forces - mucus, IgA secretion, cell desquamation and mechanical cleansing forces such as ciliary movement and bladder emptying - are entirely of host origin. In the digestive tract, however, the indigenous anaerobic microflora plays a key role, although colonization resistance-determining host factors comparable to those in the other two systems are also present. They include saliva flow and the presence of antimicrobial components such as peroxidase and lactoferrin in saliva, integrity of the mucosa, mechanical cleansing by muscular action and swallowing, peristalsis, mucus, desquamation of mucosal cells and secretory IgA.

1.6.6 Selective decontamination of the digestive tract

Following the work of van der Waaij and colleagues [4,5,60-63] it became widely accepted that the indigenous anaerobic flora of the digestive tract plays a crucial role in preventing intestinal colonization with potential pathogens [64]. The indigenous anaerobic flora is susceptible to a number of antibiotics. It is possible that colonization by potentially pathogenic bacteria (AGNB) can occur only when such bacteria find a "foothold" in the mucosa vacated by the protective anaerobes [61]. Severe illness and/or broad-spectrum antibiotic treatment may result in an increase in the size and number of these footholds by their adverse effect on the anaerobic bacteria. The adherence of larger numbers of potentially pathogenic bacteria (colonization) appears to be associated with detectable penetration of the mucosal lining and migration into the lymphatic system (translocation) [65]. As a result empirical antimicrobial therapy was used to eliminate intestinal AGNB known to be potential pathogens while maintaining the anaerobic microflora in the management of immunosuppressed patients [47,48,66]. The technique was originally described as SDD [60].

It was against this background that the Dutch team in Groningen [3] carried out the first clinical trial of SDD supplemented with parenteral cefotaxime in the novel setting of the ITU and started the vigorous controversy which persists today [11,43,67,68].

1.7 The ß-lactam antibiotics

1.7.1 Introduction

One of the most important group of antibiotics both historically and medically is the ß-lactam group to which cefotaxime belongs. Lactam, from lact(one) and am(ide), is the chemical term for any of a group of inner amides derived from amino acids, having the characteristic group -CONH-. A lactam is a cyclic amide: "ß" indicates that the amine group used to form the amide is on the second carbon from the carbonyl (C=O) end. The ß-lactam structure is therefore a four-membered ring. Such a small ring is normally a strained structure of low inherent stability. This explains why some of the penicillins readily undergo acid or base hydrolysis [69]. The ß-lactam antibiotics include the penicillins, cephalosporins, cephamycins, monocyclic ß-lactams (monobactams) and carbapenems. These antibiotics are called ß-lactams because they contain the ß-lactam ring system. Their antibacterial activity depends on the integrity of the ß-lactam ring structure; once disrupted, antibacterial activity is lost. It was recognized in 1949 [70] that the lability of one link in the penicillin molecule was the single most important factor in all of the reactions of that substance. That link was the amide link of the ß-lactam ring structure. In the best-known ß-lactam agents, the penicillins and cephalosporins, the ß-lactam ring is fused to a five-membered thiazolidine ring or six-membered dihydrothiazine ring respectively (Figure 1).

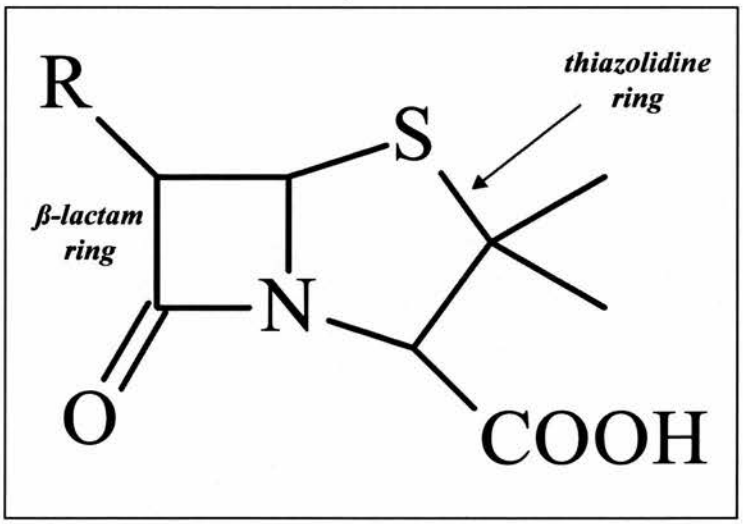

Figure 1a The basic structure of a penicillin.

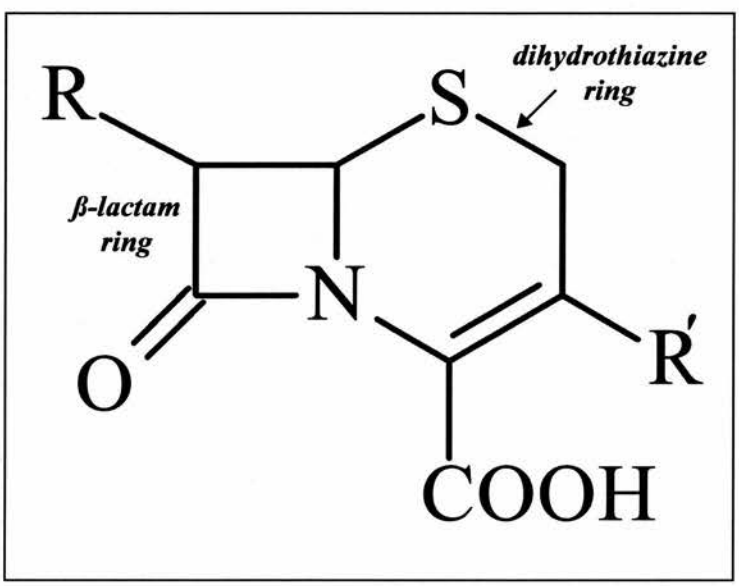

Figure 1b The basic structure of a cephalosporin.

The antimicrobial activity of the ß-lactam antibiotics results at least in part from their ability to interfere with the synthesis of the peptidoglycan component in the bacterial cell wall. Peptidoglycan has a mesh structure with polysaccharide strands cross-linked by peptide bridges and has a vital role in maintaining the strength of the cell wall. ß-Lactam antibiotics inhibit the penicillin-binding proteins (PBPs) which structurally resemble and are probably derived from serine proteases [71]. These PBPs are transpeptidases, carboxypeptidases and endopeptidases that catalyse the cross-linking of the peptidoglycan [72,73]. PBPs are capable of binding to ß-lactams because the highly stressed amide group of the ß-lactam ring is conformationally similar to the D-alanyl-D-alanine bond of the peptidoglycan pentapeptide [74,75]. As a result the PBPs react with the ß-lactams instead of their natural substrates and yield stable covalent esters that lack catalytic activity. The mechanisms are complex and many subtleties are incompletely understood. In simplistic terms the ß-lactams prevent the formation of peptide bridges causing a weakened wall to be produced. They may also activate cell wall degrading enzymes called autolysins [76,77].

1.7.2 Penicillins

Penicillin was discovered in 1928 and isolated in 1929 from *Penicillium notatum* by Alexander Fleming [78]. In 1940 the systematic study of the chemistry and production of penicillin by Florey, Chain and associates began in Oxford and in 1941 the first clinical trials were instigated. As Britain was in the throes of World War II the commercial production of penicillin was initiated in the USA. There followed a unique, international collaborative effort to ascertain the chemical constitution of penicillin and to devise methods for its synthesis [79].

According to Johnson and colleagues [70] the first evidence interpreted specifically in favour of the ß-lactam structure was that discovered by the Merck group in 1944 when they succeeded in removing the sulphur from benzylpenicillin. The properties of the resulting desthiopenicillin strongly suggested its ß-lactam

structure [80]. This was followed by cogent theoretical advocacy of the ß-lactam structure by Johnson and colleagues [70]. Finally, the results of X-ray crystallography studies at Oxford and Greenwich in 1945 and 1946, as documented by Crowfoot and colleagues [81], provided indisputable evidence for the existence of the four-ring ß-lactam structure. In 1945 Fleming, Florey and Chain shared the Nobel Prize for their pioneering work on penicillin.

The penicillin nucleus, 6-aminopenicillanic acid (6-APA) was itself produced first as a natural product [82] but was subsequently obtained more easily from penicillin G by removal of the side chain, either by chemical or enzymatic means [83]. Its isolation enabled the synthesis of a variety of penicillin compounds by coupling the free 6-amino group of the penicillanic acid to free carboxyl groups of different radicals.

1.7.3 Cephalosporins

The cephalosporins originate from the work of Giuseppe Brotzu, a Professor of Bacteriology in the University of Cagliari, Sardinia in the mid-1940's. Brotzu hypothesized that the apparent clearing of microorganisms from the water in the vicinity of a sewage outlet in the harbour at Cagliari might result from the inhibitory effect of substances produced by another microorganism. His search led to the isolation of a fungus, *Cephalosporium acremonium*, the first microbial source of a cephalosporin [84].

In 1948, unable to proceed further with this work, Brotzu sent a culture of the organism to Sir Howard Florey at Oxford where workers led by Professor Edward P Abraham proceeded to identify several fermentation products of *Cephalosporium acremonium* which possessed antimicrobial activity. In 1953 they identified cephalosporin C [85] which became the foundation upon which current cephalosporin antimicrobials are constructed.

Cephalosporin C is a ß-lactam compound in which the ß-lactam ring is fused to a six-membered dihydrothiazine ring, in contrast to the penicillins where the comparable unit is a five-membered thiazolidine ring. The knowledge that substitution of different side chains at the 6-acylamino site of the 6-APA resulted in semisynthetic penicillins of increased potency set the stage for attempts to modify cephalosporin C and thereby enhance its potency [86]. The structure of cephalosporin C, and its relationship to penicillin, was established [87] shortly after the discovery and isolation of 6-APA and at about the same time as the introduction of the first of the semisynthetic penicillins. Acid hydrolysis of the side chain of cephalosporin C yielded a nucleus, 7-aminocephalosporanic acid (7-ACA) from which the cephalosporins have been derived as semisynthetic compounds in a manner analogous to that of the semisynthetic penicillins.

1.7.4 Classification of cephalosporins

The most widely used classification system combines the parenteral and oral cephalosporins into generations based on their spectrum of microbiological activity [88]. The first-generation compounds have a relatively narrow spectrum of activity focused primarily on the Gram-positive cocci but with some activity against Gram-negative bacteria. The second-generation cephalosporins have variable activity against Gram-positive cocci but have increased activity against Gram-negative bacteria. The cephamycins (7-α-methoxycephalosporins) were produced from *Streptomyces* species [84]. In spite of relatively increased activity against Gram-negative aerobic and anaerobic bacilli the cephamycins are included in the second generation. Third-generation cephalosporins (e.g. cefotaxime and ceftazidime) are those with very marked activity against aerobic Gram-negative bacteria. Some of these compounds have limited activity against Gram-positive cocci, particularly methicillin-susceptible *Staphylococcus aureus*.

1.7.5 Cefotaxime

Cefotaxime is a third-generation cephalosporin resulting from the addition of an aminothiazole group to the acyl side chain at position 7 and the addition of an iminomethoxy group to the α-site of this side chain (Figure 2).

The important structural feature of this aminothiazolyloxime-cephalosporin is the conjunction in the acetyl side-chain of the aminothiazolyl ring and the α-*syn*-methoxyimino group [89]. Oxime derivatives exist as two geometrical isomers, the *syn* form and the *anti* form. In the cephalosporin series it was known that whereas the *syn* oxyimino derivatives retained the antibiotic properties of the parent molecule and had increased resistance to the deactivating action of the ß-lactamases, the corresponding *anti* oximes had inferior antibiotic properties when compared to the parent molecule. This was found to be the case with cefotaxime which is the *syn* oxime derivative. The aminothiazolylmethoxy side chain confers stability to many of the ß-lactamases of Gram-negative bacteria while retaining high activity against streptococci and to a lesser degree against methicillin-susceptible *Staphylococcus aureus* [88].

1.7.6 Resistance to the ß-lactam antibiotics

Resistance to the ß-lactam antibiotics can be mediated through three mechanisms: decreased ability of the antibiotic to reach its PBP target, alteration of a PBP target that is essential for cell survival, or production of ß-lactamases that inactivate the antibiotics [88].

In Gram-negative bacteria the outer membrane, in addition to concentrating ß-lactamases within the periplasm, also contributes to resistance because it acts as a barrier to the penetration of ß-lactam compounds to target PBPs [90].

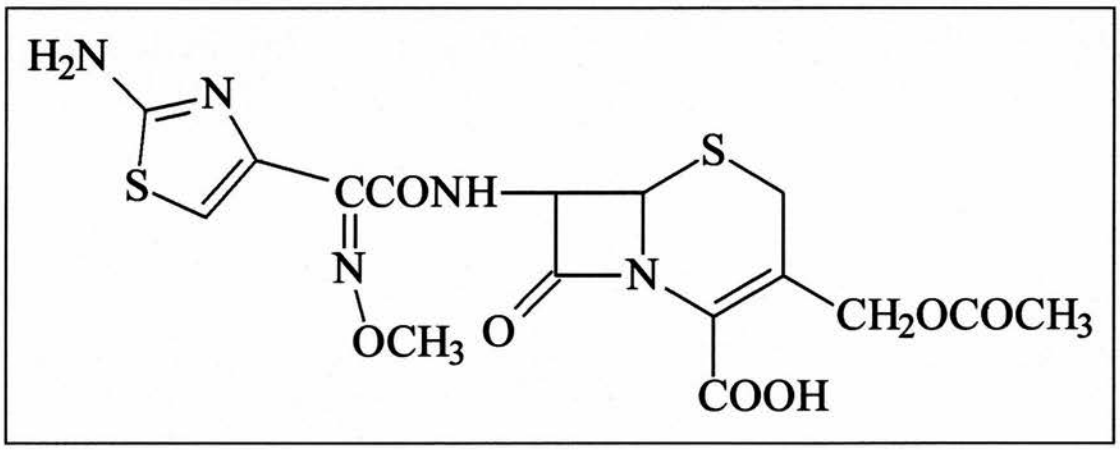

Figure 2 The structural formula of cefotaxime.

ß-Lactam compounds that conform to the structure and charge requirements of the porin channels can penetrate into the bacterial cell and, if they are unaffected by any ß-lactamase that is present, bind to target PBPs [91,92]. The activity of ß-lactams that intrinsically penetrate slowly may be limited if they are susceptible to hydrolysis or to removal by high concentrations of ß-lactamases in the periplasmic space. Mutations that alter porin protein structure may render a strain that was initially susceptible to a given ß-lactam antibiotic, resistant. It is thought unlikely that porin permeability barriers alone should result in the resistance of Gram-negative bacteria to ß-lactam antibiotics [93]. Nevertheless the rate of penetration of a ß-lactam antibiotic through the outer membrane is intrinsically linked to resistance mediated by ß-lactamases [94].

Although multiple mechanisms often work in concert, ß-lactamase destruction of antibiotic is undoubtedly the most important [95]. ß-Lactamases are enzymes that hydrolyze the ß-lactam ring of the ß-lactam antibiotics rendering them inactive.

1.8 "Penicillin- and cephalosporin-destroying enzymes" and ß-lactamases

An enzyme from bacteria capable of destroying penicillin was described by Abraham and Chain in 1940 [96], before the widespread use of penicillin in the treatment of bacterial infections. In fact Fleming had been the first to note, in 1929, that certain groups of bacteria such as the "coli-typhoid" group were not inhibited by penicillin [78]. In 1940 Abraham and Chain [96] pursued Fleming's observation by crushing a suspension of *Escherichia coli* "*B. coli*" in a mill to produce an extract which destroyed the growth-inhibiting properties of penicillin. Heat and papain digestion inactivated the extract leading the investigators to conclude that the active component was an enzyme which they named penicillinase. The paper ended with an apocryphal statement:

"The fact that a number of bacteria contain an enzyme acting on penicillin points to the possibility that this substance may have a function in their metabolism" [96].

At that time the chemical structure of penicillin had not yet been worked out and the nature of the reaction catalysed by penicillinase was unknown. In 1943 the ß-lactam structure was proposed [97] and later the existence of the ß-lactam ring was found to be indisputable [70]. In 1944 it had been concluded that penicillinase converted penicillin to penicilloic acid [97]. Interest in penicillinase developed rapidly. Nearly all of the early work concerned Gram-positive bacteria such as *Bacillus* species and staphylococci. Until the 1960s most attention focused on staphylococcal penicillinase as the prevalence of penicillin-resistant staphylococci rapidly reached epidemic proportions in hospitals where penicillin was used widely, then gradually spread into the community [98].

Reviewing penicillinase in 1960 Pollock [99] thought it reasonable to suppose that the majority, if not all, of the preparations referred to as "penicillinase" owed their activity to an enzyme which should be regarded as a "penicillin-ß-lactamase" catalysing the hydrolysis of penicillins to penicilloic acid in a reaction similar to their chemical breakdown by dilute alkali i.e. hydrolysis of the amide bond in the ß-lactam ring of the penicillin molecule. Almost immediately after cephaloridine came into clinical use in 1964 it was realised that certain ß-lactamases (e.g. from indole-positive *Proteus* species and *Enterobacter* species) were more active against cephalosporins than against penicillins. This finding was predictable in view of the work by Newton and Abraham on the degradation of the natural product cephalosporin C [100]. The nomenclature became confusing as enzyme preparations were termed variously penicillinases, cephalosporinases and ß-lactamases.

Citri and Pollock [101] rationalised the situation in 1966 by defining ß-lactamase as an enzyme which hydrolyzed the amide bond in the ß-lactam ring of 6-APA or 7-ACA and/or in their *N*-acyl derivatives (penicillins being *N*-acyl derivatives of 6-APA and cephalosporins being *N*-acyl derivatives of 7-ACA). While acknowledging that the ability to hydrolyze cephalosporin relative to benzylpenicillin might be one of the distinctive properties of an enzyme, they thought that the general term ß-

lactamase appeared to be appropriate even in the extreme cases when only one of these substrates was measurably hydrolyzed [101].

While these early discoveries about ß-lactamases were being made knowledge of bacterial genetics was also expanding. It was soon recognised that bacterial genetics and antimicrobial resistance mechanisms were inextricably linked.

1.9 Genetics of antibiotic resistance

1.9.1 Introduction

Genetic variability allows microbial evolution to occur. Antimicrobial agents exert strong selection pressures on bacterial populations favouring those organisms that are capable of resisting them [102,103]. Genetic variability may occur by a variety of mechanisms: 1) Point mutations may occur in a nucleotide base pair. These mutations may alter the target site of an antimicrobial agent, interfering with its activity. 2) Whole-scale rearrangement of large segments of deoxyribonucleic acid (DNA) from one location on the bacterial chromosome to another may occur as a single event. These rearrangements are frequently created by specialised genetic elements known as transposons or insertion sequences which have the capacity to move independently from the rest of the bacterial chromosome [103]. 3) Bacteria may acquire foreign DNA carried by plasmids, bacteriophages or transposable genetic elements. Inheritance of these extrachromosomal elements contributes to an organism's ability to withstand selection pressures imposed by antimicrobial agents [104] and endows bacteria with a seemingly unlimited capacity to develop resistance to antimicrobial agents. Once an antibiotic resistance gene evolves, this resistance determinant may spread to other bacteria by transformation, transduction, conjugation or transposition. Favoured clones of bacteria may then proliferate in the flora of patients who receive antibiotics [105]

1.9.2 Plasmids

Plasmids are extrachromosomal genetic elements usually made of circular double-stranded DNA molecules that range in size from less than 10 kilobase pairs (kb) to greater than 400 kb and are very common in bacteria. They are autonomous, self-reproducing elements that require an origin for replication and regions that facilitate their stable maintenance in host bacteria [106]. Conjugative plasmids require additional genes that can initiate self-transfer [107]. It has been shown that extrachromosomal genetic elements were present in bacteria prior to the advent of antibiotics [104] but the introduction of antibiotics into clinical medicine has created selection pressures which favour the dissemination of antibiotic resistance by mobile genetic elements (e.g. plasmids, transposons and other mobile genes). Rapid increases in the spread of antibiotic resistance within and between species often correlate with the dissemination of specific resistance (R) genes.

The generic term "plasmid" was introduced by Lederberg in 1952 [108] for any extrachromosomal genetic particle. He intended to clarify the classification of agents that until then had been:

> "thought of disjunctively as parasites, symbionts, organelles, or genes. ..... It was important to have a new term, bereft of confusing baggage. ..... The term plasmid was then invented as a hybrid of cytoplasm or plasmagene and '-id', as in plastid, chromatid, or id (Weissmann, Freud, Latin 'it')." [109].

From about 1970 plasmids became important agents in molecular genetic research and biotechnology. They were also recognized as playing a pivotal role in the evolution of microbial resistance and pathogenicity. The first plasmid to be identified was the *Escherichia coli* fertility factor F which was discovered because of its ability to mediate transfer of chromosomal markers from one strain of *Escherichia coli* to another. Sexual differentiation was attributed to possession of a transmissible factor called F (for fertility) with F^+ cells acting as donors of information and F^- cells as recipients [110]. The physical nature of F was unknown

at that time. It could spread through bacterial populations but was not apparently part of the bacterial chromosome [110,111]. In enterobacteria the identification of F was followed by the discovery of two other major groups of plasmids, the colicinogenic (Col) plasmids and the infectious drug-resistance plasmids [112].

The probability that plasmids were composed of DNA was suggested by the finding that F factors, R factors and Col factors could be inactivated by the decay of radioactive ^{32}P incorporated in their host cells [113-115]. Direct demonstration that the F factor was made of DNA came from the work of Marmur and colleagues [116] who isolated it on a caesium chloride density gradient. This laid the basis for future quantitative studies.

1.9.3 Transposons

Transposable elements are discrete sequences of DNA that can move from one genetic locus to another, on the same or on a different replicon, by a process of recombination called transposition [102]. The term "transposable element" was used originally to define mobile genetic elements in wheat [117]. The size of these elements can vary markedly and many different properties can be transposable. Transposable elements retain their structural integrity during transposition and the recombination does not require DNA base sequence homology between the donor and recipient genetic loci i.e. the recombination is independent of the bacterial *rec-A* system. This usually results in whole-scale modifications of large sequences of DNA as a single event [102,118]. When a transposable element contains an accessory gene, encoding some marker, it is called a transposon. The transposable markers may be antibiotic resistance determinants, toxins and other virulence factors, or metabolic functions. According to Kopecko [102], cataloguing of the properties of many transposons detected on plasmids obtained from a large variety of different bacteria clearly demonstrated the fact that plasmids were evolving through a process

of exchange of discrete transposable units. Transposable elements have been found in many species of bacteria, Gram-positive as well as Gram-negative.

1.9.4 Conjugation

Conjugation is the process whereby DNA is transferred from one bacterial cell to another by a mechanism that requires cell to cell contact [119]. The information required for transfer is encoded on transfer-proficient plasmids which may also be able to promote the transmission of mobilizable plasmids and even chromosomal genes. Conjugation gives the subset of genes usually located on plasmids an evolutionary advantage. As conjugation is essentially a replication process it allows plasmids to replicate more frequently than the chromosomal genes and enables them to transfer to alternative bacterial hosts [120]. If cells which receive the plasmid are favoured by natural selection, the plasmid will spread rapidly. This is illustrated by the world-wide appearance of antibiotic-resistant bacteria.

Apparently conjugation can be divided on both physiological and genetic bases into two parts: the recognition of recipient cells by donor cells that leads to mating pair formation and the subsequent physical transfer of plasmid DNA [120]. Conjugative plasmids from Gram-negative bacteria control the formation of an extracellular pilus which has an essential role in the recognition of recipient cells and the establishment of cell-to-cell contact. Bradley [121] differentiated three major groups of sex pili according to their morphology on electron microscopy. After the initial contact between the tip of the pilus and the recipient cell the pilus retracts. Pilus dissassembly is thought to be crucial in establishing contact between the donor and recipient cell surfaces and the formation of a DNA transport pore [122 {p98}]. Initiation of DNA transfer takes place at a specific site on the DNA called the origin of transfer [123]. Plasmids usually specify a surface (or entry) exclusion system that prevents the unproductive transfer of the plasmid to a cell that already possesses a copy [120].

1.9.5 Infectious drug resistance and the link with plasmids or "R factors"

Resistance to antibacterial agents transferable by conjugation, i.e. from one cell to another by direct contact, was discovered in the *Enterobacteriaceae* in Japan in 1959 [124-128] and later reported in Britain in 1962 [129] and in Germany in 1963 [130]. "Contagious" drug resistance was often multiple, rendering organisms simultaneously resistant to several (as many as seven) unrelated antibacterial drugs of therapeutic importance [131].

After the World War II the use of antibacterial drugs to combat infection became increasingly common. It was the epidemiology of drug resistance in shigellae that led Japanese bacteriologists to postulate that multiple drug resistance might be transferred from one bacterial species to another. In the early 1960's the greater part of scientific knowledge, both in epidemiology and in the fundamental analysis of infectious drug resistance, came from Japanese workers [131].

From 1956 onwards shigellae resistant to streptomycin, tetracycline, chloramphenicol and sulphonamides were isolated with increasing frequency in Japan. In dysentery outbreaks, some of the shigellae isolated were sensitive to all drugs tested, while others of the same serotype were multiply resistant. Patients excreting drug-sensitive shigellae, when treated with only one antibacterial drug, subsequently excreted multiply-resistant shigellae. In some studies multiply-resistant *Escherichia coli* were also isolated from the patients with dysentery [126]. No acceptable explanation of these findings was forthcoming until Akiba [132] suggested that transfer of multiple drug resistance from resistant *Escherichia coli* to sensitive shigellae might occur in the patients' intestinal tracts. This transfer was finally demonstrated in mixed culture independently by Ochiai and colleagues in 1959 [124] and by Akiba and colleagues in 1960 [125]. Although the commonest transmissible resistance pattern in Japan was against streptomycin, tetracycline, chloramphenicol, and sulphonamide, combinations of resistance to three or two of

these drugs, as well as single drug resistance, were also found to be transferable. After these researchers [124,125] succeeded in discovering the transfer of multiple drug resistance *in vitro*, they attempted unsuccessfully to transfer the resistance with cell-free filtrates of resistant donor cultures. They therefore concluded, according to Watanabe in his review of the Japanese language literature [128], that cell-to-cell contact (or conjugation) was essential for the transfer. This finding had been confirmed by Mitsuhashi and colleagues [126]. The transfer of multiple drug resistance was not found to alter the basic diagnostic biochemical or serological markers of the organisms [124,125].

The analogy had been drawn between the agents of such transferable drug resistance and the sex factor F of *Escherichia coli* K12 [126]. A few years earlier Lederberg and colleagues [110] and Hayes [111] had found that F, the causative agent of gene transfer between strains of *Escherichia coli*, was unlikely to be part of the chromosome and could spread through bacterial populations. Mitsuhashi and colleagues [133] reported that multiple drug resistance could be transferred among the substrains of *Escherichia coli* K12 irrespective of their sexuality (presence or absence of F factor), indicating that the F factor of K12 was not required for the transfer of multiple drug resistance. This fact was confirmed by Watanabe and Fukasawa [127] who found also that the responsible resistance factors were transferred in mixed culture independently of the host chromosome, using F^- strains of *Escherichia coli* K12 with various chromosomal markers. Multiple drug resistance could therefore be regarded as a kind of "infective inheritance", a phenomenon which was first described in microorganisms by Griffith [134]. "Infectious resistance" to streptomycin, tetracycline and sulphonamide was found in 15 of 306 cultures of *Salmonella typhimurium*, phage type 27, which had been isolated during an outbreak of gastroenteritis in a London hospital in 1959 [129]. This work was in fact the first Western description of transmissible antimicrobial resistance.

The infectious agents which mediated the resistance came to be known as R factors and were shown to be extrachromosomal genetic elements, consisting of DNA [128,135]. In his review in 1963 Watanabe [128] classified R factors as episomes, acknowledging that by definition a chromosomally integrated state had to be demonstrated for their inclusion in this category as originally defined [136]. Watanabe suggested that R factors had arisen in enteric bacteria by the attachment of part of the host's genome to a sex factor, the resistance transfer factor (RTF) [128].

It had been found that R factors could be transferred *in vitro* between cells of many genera: all genera of the *Enterobacteriaceae* [137] as well as other Gram-negative bacilli such as *Vibrio* species [138], *Serratia* species [139] and *Pasteurella* species [140] and evidence for *in vivo* R factor-mediated resistance in Britain, Europe and elsewhere began to accumulate [131]. In 1965 Datta [131] contended that although R factors were frequently referred to in published works as episomes, they had not been proved to be chromosomally integrated and thought it preferable to refer to R factors as plasmids [108].

In 1965 Anderson and Datta [141] reported that several strains of *Salmonella typhimurium* phage type 1a isolated in England in 1962 from humans and pigs were resistant to ampicillin and penicillin. This was the first published observation of such resistance in wild strains of *Salmonella* species. Resistance to ampicillin was always part of a multiple resistance pattern and was readily transferable to *Escherichia coli*. Resistance to tetracycline was invariably associated with resistance to ampicillin and feeding materials for pigs commonly included penicillins and tetracyclines. Anderson and Datta [141] suggested that the use of these antibiotics as feeding additives in livestock might have been responsible for the emergence of resistance to penicillins and tetracyclines in the *Salmonella* strains. As resistance to ampicillin was associated with the production of a penicillin-destroying enzyme, the inference was that the acquisition of an R factor resulted in the production of the

enzyme. Anderson and Datta [141] drew the analogy between these Gram-negative bacilli and *Staphylococcus aureus*, in which penicillinase production, according to Harmon and Baldwin [142], seemed to be controlled by an extrachromosomal genetic element .

1.9.6 Plasmids or "R factors" and the link with ß-lactamases

The finding that the genetic information for resistance to penicillins, including ampicillin, could be carried on R factors was described independently in Britain [141], Greece and Switzerland [143]. This was to have far-reaching consequences as it predicted that ampicillin resistance would spread not only among strains of *Escherichia coli* but also to other genera. In 1965 Datta and Kontomichalou [143] were able to report that this penicillin-destroying enzyme was penicillinase because it liberated penicilloic acid on incubation with benzylpenicillin. The evidence was therefore presented that the genetic information for the biosynthesis of penicillinase was carried on an R factor [143]. One of the R factors studied was R_{TEM} carried by *Escherichia coli* strain TEM which had been isolated by Kontomichalou [143] from a patient called Temoniera [98] in Athens in 1963. *Escherichia coli* strain TEM synthesised relatively large amounts of penicillinase and provided a convenient opportunity to attempt for the first time the study of a purified rather than a crude preparation of a penicillinase from a Gram-negative species [144]. The gene responsible for penicillinase synthesis in *Escherichia coli* strain TEM was also found to be carried on an extrachromosomal R factor [143].

A number of ß-lactamases from Gram-positive species, *Bacillus cereus* [145], *Bacillus licheniformis* [146], and *Staphylococcus aureus* [147,148], had been purified and their properties examined but until 1966 no penicillinase from Gram-negative species had been purified to any extent [144]. The enzyme from *Escherichia coli* strain TEM showed little resemblance to the penicillinases of the Gram-positive species. The fact that the penicillinase from *Escherichia coli* strain

TEM could be liberated from the bacteria under conditions in which the cytoplasmic membrane was protected [149] suggested that penicillinase might be one of the periplasmic enzymes of *Escherichia coli* [144].

Datta and Richmond [144] thought it likely that penicillinase genes in Gram-negative species could either be chromosomal or carried on R factors and in view of the infectivity of R factors among the *Enterobacteriaceae* cautioned that no penicillinase should be regarded as species-specific until its synthesis was proved to be controlled by chromosomal genes These discoveries accelerated interest in the ß-lactamases of Gram-negative bacilli and the search for ß-lactam antibiotics resistant to these ß-lactamases became a major thrust of the pharmaceutical industry.

1.10 ß-Lactamases of Gram-negative bacteria from the 1960s onwards

1.10.1 Introduction

Resistance to ß-lactam antibiotics is due mainly to the production of ß-lactamases, enzymes that inactivate these antibiotics by splitting the amide bond of the ß-lactam ring. ß-Lactamases of Gram-negative bacteria are cell-associated enzymes located in the periplasmic space that lies between the inner cytoplasmic membrane and outer lipopolysaccharide membrane. Therefore these ß-lactamases are concentrated and strategically placed to protect the target PBPs (located on the outer surface of the inner cytoplasmic membrane of the cell envelope) from exposure to ß-lactam antibiotics. Most of the enzymes, like the PBPs themselves, belong to a family of serine proteases. ß-Lactamases of Gram-negative bacteria may be encoded either by chromosomal genes or by transferable genes located on plasmids or transposons. These enzymes may be constitutive (always active) or inducible (capable of being activated by another agent), with affinity for penicillins or cephalosporins or both types of compounds.

1.10.2 Classification of β-lactamases

The first attempts to classify the ß-lactamases from Gram-negative bacteria were made by Ayliffe [150] soon after ampicillin was first introduced into clinical use. Both enzymes studied initially were "penicillinases" but soon afterwards Fleming and colleagues [151] described a ß-lactamase predominantly active against cephalosporins. Since then such a wide variety of different ß-lactamase profiles were detected in various species of enterobacteria and pseudomonads that a simple classification into penicillinases and cephalosporinases was no longer of much value. Indeed there seemed to be an almost continuous spectrum of properties extending from extreme cephalosporinases on one hand to enzymes predominantly active against penicillins on the other [152].

1.10.2.1 Richmond and Sykes (1973)

One of the first widely used classification schemes, proposed by Richmond and Sykes in 1973 [152], grouped the ß-lactamases of Gram-negative bacteria into five main classes by an extension of the scheme suggested previously by Jack and colleagues [153] and Richmond and colleagues [154] which had recognised four main classes. The enzymes were classified mainly on the basis of substrate profile and inhibition studies and provided the first broadly useful frame of reference [152].

Class I enzymes, subdivided into types a, b, c and d, were predominantly cephalosporinases (with a high rate of hydrolysis of cephalosporins) and included the chromosomally determined ß-lactamases of most of the *Enterobacteriaceae* and pseudomonads. Class II enzymes, subdivided into types a and b were penicillinases, active mainly against penicillins. Class III enzymes, having only one subtype "a", showed broad-spectrum activity (approximately equal activity against penicillins and cephalosporins) and were sensitive to inhibition by cloxacillin but resistant to inhibition by *para*-chloromercuribenzoate (*p*CMB). Class IV enzymes, subdivided into types a, b and c were also broad-spectrum enzymes but resistant to inhibition by

cloxacillin and sensitive to inhibition by *p*CMB. Class V enzymes, the fifth group suggested by Richmond and Sykes and subdivided into types a, b, c and d were penicillinases able to hydrolyze cloxacillin and resistant to *p*CMB inhibition. The heterogeneity within some of these classes and the problems of determining parameters such as enzyme inhibition by *p*CMB using crude enzyme preparations made it difficult to utilise this classification, especially when additional information on properties such as inducibility of enzyme production or location (chromosome or plasmid) of the ß-lactamase genes was lacking.

1.10.2.2 Sykes and Matthew (1976)

In 1976 Sykes and Matthew [155] catalogued all the ß-lactamases described to date. Recognising that the diversity of ß-lactamases would become as extensive as that of bacteria, they thought that a formally divided classification scheme had become unrealistic. Instead they grouped ß-lactamases first into those that were normally mediated by the bacterial chromosome or by a transmissible R plasmid and second by substrate profile, hoping that this grouping would be useful clinically. The chromosomal enzymes were subdivided into those that were primarily penicillinases, primarily cephalosporinases, or broad-spectrum ß-lactamases corresponding to Classes II, I and IV respectively of Richmond and Sykes [152]. The R plasmid-mediated ß-lactamases were subdivided into the broad-spectrum "TEM type" of enzyme and other enzymes that hydrolyzed isoxazolyl ß-lactam substrates, corresponding to Classes III and V respectively of Richmond and Sykes [152]. By 1975, using isoelectric focusing, ß-lactamases had been found in 354 strains of Gram-negative bacteria from 19 genera [156]. Because of the extensive work by Matthew and colleagues [156,157] isoelectric focusing became one of the most critical means of characterising ß-lactamases. A major feature of the Sykes and Matthew compilation [155] was the inclusion of the isoelectric point (pI) for many of the clinically important ß-lactamases.

1.10.2.3 Ambler (1980)

Until 1980 classifications of ß-lactamases had been based on their functional characteristics. In 1980 Ambler [158] pointed out that structural information, as well as being able to provide evidence about the origin of the ß-lactamases, was necessary for the elucidation of their mechanism of action and proposed yet another new nomenclature based on molecular structure. At that time only four amino acid sequences of ß-lactamases were known. A single class of serine enzyme was designated, the class A ß-lactamases that included the *Staphylococcus aureus* PC1 penicillinase, in contrast to the class B metallo-enzyme from *Bacillus cereus*. The class C ß-lactamases were described by Jaurin and Grundström [159] in 1981. The class D oxacillin-hydrolyzing ß-lactamases were segregated from the other serine enzymes in the late 1980s [160,161].

There has always been controversy over the physiological role of ß-lactamases in the life history of the bacteria which produce them [96,152,155,162]. Homology was found between the amino acid sequence around the penicillin-binding sites of the D-alanine carboxypeptidases of the Gram-positive bacteria *Bacillus stearothermophilus* and *Bacillus subtilis* and the active site serine of these class A ß-lactamases, supporting the hypothesis that these ß-lactamases might be derived from PBPs involved in peptidoglycan synthesis [163,164]. Hybridization techniques using DNA probes were increasingly used during the 1980s to study the molecular basis for ß-lactamase diversity [165].

1.10.2.4 Bush (1989)

Deficiencies in the available ß-lactamase classification schemes became apparent during the 1980s and a modification of the Richmond and Sykes scheme was proposed by Bush in 1988 [165]. A major reorganization took place. Using substrate and inhibitor profiles in addition to physical characteristics a compilation of distinguishing ß-lactamase data was provided for both chromosomal and plasmid-

mediated enzymes [166-168]. Unlike many earlier schemes the Bush scheme did not use the location of the gene encoding the ß-lactamase as a primary classification factor. This reflected the appreciation of the mobility of ß-lactamase genes, with chromosomal genes finding their way to plasmids and vice versa.

1.10.2.5 Bush-Jacoby-Medeiros (1995)

In 1995 Bush, Jacoby and Medeiros [169] presented an updated version of the Bush scheme in which the functional characteristics of ß-lactamases based on substrate preference among penicillin, oxacillin, carbenicillin, cephaloridine, expanded-spectrum cephalosporins and imipenem were correlated with molecular structure as first proposed by Ambler [158], in a dendrogram for those enzymes with known amino acid sequences.

1.10.3 Action of ß-lactamases

A few ß-lactamases utilise zinc ions to disrupt the ß-lactam ring but a far greater number operate via the serine ester mechanism [170] as shown in Figure 3. The enzyme first associates noncovalently with the antibiotic to yield the noncovalent Michaelis complex. The ß-lactam ring is then attacked by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester. Hydrolysis of the ester finally liberates active enzyme and the hydrolyzed, inactive drug. This mechanism is followed by enzymes of molecular classes A and C, but class B enzymes utilise a zinc ion to attack the ß-lactam ring [170,171].

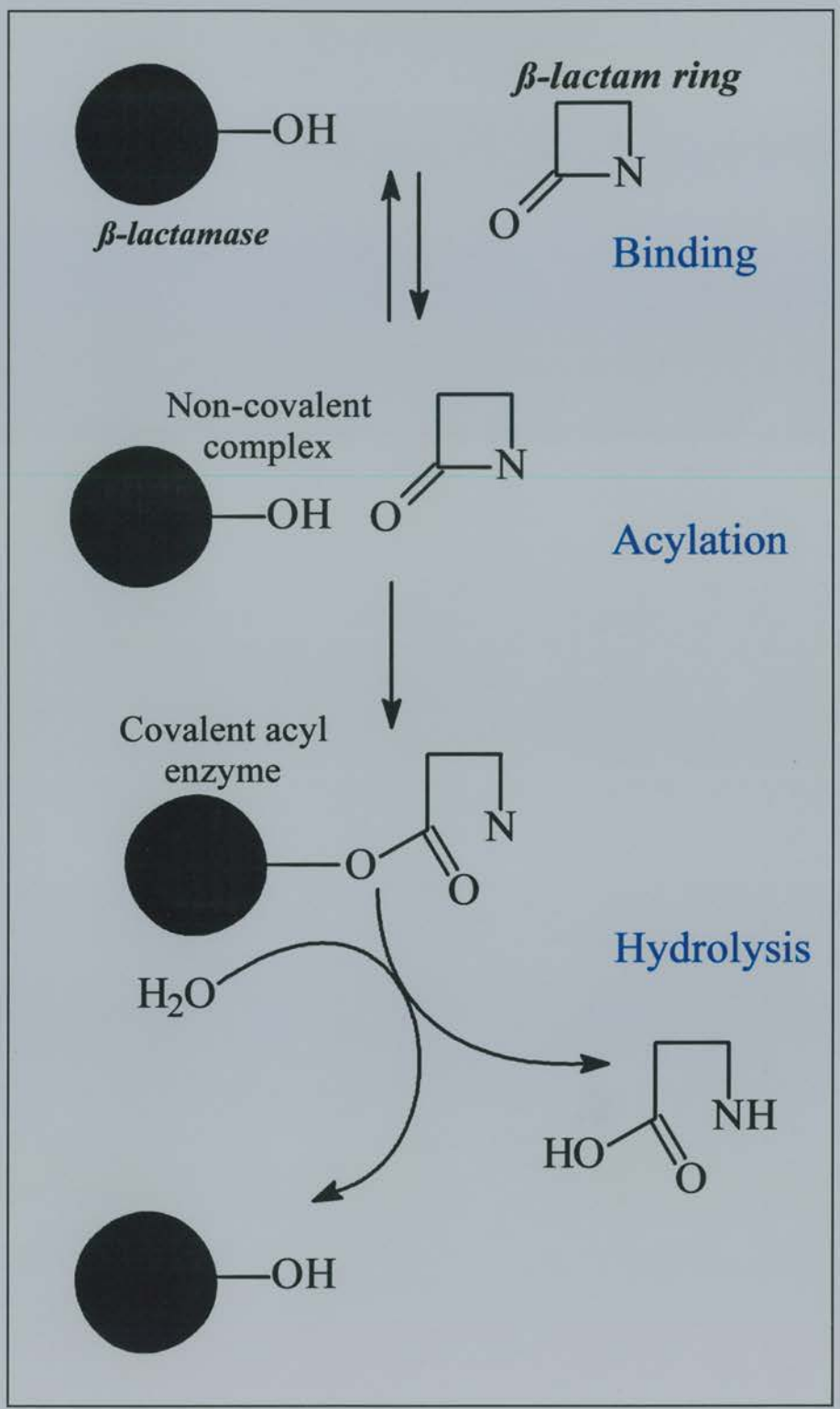

Figure 3 The action of a serine ß-lactamase [170,171].

1.10.4 Distribution of ß-lactamases in Gram-negative bacteria

1.10.4.1 Chromosomal enzymes

Matthew and Harris [157] found that nearly all Gram-negative bacteria produced some chromosomally-determined ß-lactamase and that the ß-lactamases produced by strains from one genus always had different pIs from those produced by strains from another genus. This was true even when the genera were fairly closely related taxonomically and produced enzymes with similar specificities e.g. *Citrobacter*, *Enterobacter* and *Serratia* whose enzymes are primarily cephalosporinases inhibited by cloxacillin and methicillin. Matthew and Harris [157] also found that different species from the same genus always produced different ß-lactamases and that the ß-lactamases were species-specific. The pIs did not appear to be related to the biochemical properties of the enzymes, such as the substrate profiles or susceptibility to ß-lactam inhibitors that formed the basis of the Richmond and Sykes (1973) classification [152]. Enzymes might have similar biochemical properties and differ greatly in isoelectric focusing; alternatively they might have very different biochemical properties and yet focus close to one another [157]. Although Matthew and Harris [157] suggested that nearly all Gram-negative bacteria coded for a species-specific chromosomally-mediated ß-lactamase, several different chromosomal enzymes from the same strain have since been identified [166].

The regulation of the ß-lactamase gene varies in Gram-negative bacteria. *Escherichia coli* and shigellae, usually have only insignificant levels of uninducible molecular class C enzymes, often called AmpC types [172,173]. Other species produce chromosomal ß-lactamases copiously, whether constitutively or inducibly [155,166-168,173]. Examples include isolates of klebsiellae in which molecular class A enzymes are constitutive; *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Morganella morganii*, *Serratia* species, *Providencia* species,

Pseudomonas aeruginosa and other fluorescent pseudomonads, which have inducible class C enzymes; *Citrobacter diversus*, *Proteus vulgaris*, and *Burkholderia* (*Pseudomonas*) *cepacia*, which have inducible class A enzymes and *Stenotrophomonas* (*Xanthomonas*) *maltophilia*, which has an inducible class B carbapenem-hydrolysing ß-lactamase and an unsequenced Bush group 2e cephalosporinase.

1.10.4.2 Plasmid-mediated ß-lactamases

Over 75 plasmid-mediated ß-lactamases were recorded in Gram-negative bacteria by Bush and colleagues [169] for their classification scheme in 1995, although many of these enzymes were sequence variants of a few prevalent types. Since then the numbers have dramatically increased [174]. In general plasmid-mediated ß-lactamases are distinct from the chromosomal types but a few overlaps exist. In particular the SHV-1 ß-lactamase which is common as a plasmid-mediated type [175] is also a typical chromosomal ß-lactamase of *Klebsiella pneumoniae* [170]. Another example is the PSE-4 ß-lactamase of *Pseudomonas aeruginosa* which is almost always encoded by chromosomally inserted transposons [176]. Plasmid-borne genes encoding class C ß-lactamases with high sequence homology to the chromosomal ß-lactamases of *Citrobacter freundii* (BIL-1, CMY-2, LAT-1), *Pseudomonas aeruginosa* (MOX-1) and multiple Gram-negative bacilli (FOX-1) have been noted since 1988 [177]. It is possible that all plasmid-mediated ß-lactamases have chromosomal origins [178].

a) "TEM-type" ß-lactamase

Datta and Kontomichalou [143] recognized two different types of R factor-mediated ß-lactamases and one of these, determined by the R factor R_{TEM}, subsequently termed R6K [179], was later referred to as the "TEM-type" ß-lactamase [180]. Production of the TEM-type enzyme was later shown to be mediated by many different R factors [180].

b) TEM-1 and TEM-2 ß-lactamases

The enzymes previously described as "TEM type" [143,180], although uniform in their biochemical and serological reactions, were noted to be of two types by isoelectric focusing. One type, exemplified by the enzyme encoded by the plasmid R6K, previously designated R_{TEM} [179], predominated. It was specified by R factors of nearly all compatibility groups and was referred to as type TEM-1 [181]. The other, exemplified by the enzyme encoded by the plasmid RP4, was less common and was determined by a minority of plasmids within three groups. It was called TEM-2 [181].

c) OXA ß-lactamases

The other ß-lactamase recognised by Datta and Kontamichalou [143] was determined by R_{1818}, later termed R46 [182]. Cells carrying R46 differed from those which produced the TEM-like enzyme in having a lower absolute level of ß-lactamase activity against benzylpenicillin. The R46 ß-lactamase also differed from the TEM-like enzyme in hydrolyzing methicillin at a significant rate. Subsequently the R46 enzyme was shown to hydrolyze oxacillin more rapidly than benzylpenicillin [183]. It was shown later that several R factors determined the production of the ß-lactamases which resembled that of R46 and that these were less common than the TEM-type enzymes [180]. These oxacillin-hydrolysing enzymes had been divisible by biochemical tests into two main classes one of which contained two subclasses [184]. This classification was confirmed by isoelectric focusing and the enzymes were designated OXA-1, OXA-2 and OXA-3 [181].

d) SHV-1, HMS-1 and PSE-1 - PSE-4 ß-lactamases

By 1979 six additional Gram-negative plasmid-mediated ß-lactamases had been recognized as distinct enzymes [175]. The enzyme designated SHV-1, denoting "sulphydryl-variable", by Matthew and colleagues [185] but first mentioned by Pitton [186] was shown to have a substrate profile broadly similar to that of the TEM ß-lactamases, but differed from the latter in having a greater rate of hydrolysis

of ampicillin. The most remarkable property of SHV-1 was thought to be its unique response to inhibition by the sulphydryl group reagent *p*CMB, in that the hydrolysis of cephaloridine but not that of benzylpenicillin was inhibited [185]. It has since been found that a serine hydroxyl, not a sulphydryl, is the active site residue of SHV-1 [170]. The gene for SHV-1 was known to be transposon-borne [187].

The enzyme HMS-1 (Hedges, Matthew, Smith) [185] had a substrate profile resembling that of SHV-1 but it was found to hydrolyze cephaloridine at about three times the relative rate of SHV-1. In addition, HMS-1 differed from SHV-1 and from both TEM ß-lactamases in that it was sensitive to inhibition by *p*CMB whether the substrate was benzylpenicillin or cephaloridine [175]. The other four enzymes PSE-1, PSE-2, PSE-3 and PSE-4 were determined by plasmids initially thought to be *Pseudomonas*-specific and showed the property of hydrolysing carbenicillin at least as fast as benzylpenicillin [175].

e) Extended-Spectrum ß-Lactamases

ESBLs are plasmid-mediated enzymes that confer resistance to oxyimino-ß-lactams such as cefotaxime, ceftazidime and aztreonam, antibiotics that were designed to be effective against strains producing known plasmid-determined ß-lactamases. The first report of klebsiellae resistant to oxyiminocephalosporins came fro Germany in 1983 [188]. Three unique clinical isolates from the university clinic in Frankfurt produced a mutationally-altered SHV-1 ß-lactamase, designated SHV-2, that conferred resistance to cefotaxime [188,189]. The first major outbreak due to ESBL producers, specifically isolates with TEM-3 ß-lactamase, occurred around Clermont-Ferrand in 1985-1987 [19,22] and was soon followed by outbreaks elsewhere in France [190]. Strains producing ESBLs began to be reported in the United States in 1989 to 1990 after which major outbreaks occurred [191]. Most ESBLs are mutant class A oxyimino-ß-lactamases derived from the widely prevalent plasmid-determined TEM-1, TEM-2 and SHV-1 ß-lactamases and a few are derived from the plasmid-mediated OXA-10 ß-lactamase [174]. Sixty-seven TEM

(including the inhibitor-resistant enzymes), 11 SHV and four OXA variants have been described [174]. The TEM- and SHV-derived ESBLs are commonest in klebsiellae but occur also in other enterobacteria and the inhibitor-resistant TEM-derived enzymes have been found in *Escherichia coli*. The OXA-derived ESBLs have been found in *Pseudomonas aeruginosa* [174].

Many more plasmid-mediated ß-lactamases including extended-spectrum class A ß-lactamases that are not related to TEM, SHV or OXA ß-lactamases, plasmid-mediated class C ß-lactamases such as MIR-1 that confer high-level resistance to cefoxitin and other 7-α-methoxy-ß-lactams as well as oxyimino-ß-lactams and carbapenemases (both class B metallo-enzymes and unsequenced non-metallo-enzymes) have been documented [170,174].

Against this clinical and scientific background, the aim of the present work was to investigate the cefotaxime resistance mechanisms of AGNB isolated from the surveillance flora of patients in the ITU undergoing SPEAR and to assess the implications of such resistance for the continued use of this controversial prophylactic regimen. The resistance mechanisms most worrying in this context would be those involving chromosomal AmpC ß-lactamase derepression and plasmid-mediated ESBL production.

In practice the investigation would require the summation of the following information for each isolate:

- its species identification
- whether it belonged to a chromosomal ß-lactamase-inducible species or a species more commonly associated with ESBL production
- its antibiotic sensitivity pattern

- its plasmid profile

- whether its resistance to cefotaxime was transferable

- whether any transferable resistance to cefotaxime was associated with the transfer of a plasmid

- whether the isolate produced one or more ß-lactamases

- the pI value(s) of the ß-lactamase(s) produced by the isolate.

Chapter 2 Patients, Materials and Methods

2.1 Isolates and patients

2.1.1 The SPEAR isolates and their identification

As an integral part of SPEAR, the surveillance flora of ITU patients was screened routinely by disk diffusion testing for resistance to any component of the regimen (Figure 4). Over a period of two years and ten months (5/8/88 to 20/5/91), 200 consecutive isolates of AGNB showing resistance to cefotaxime or tobramycin were collected from throat swabs, tracheal aspirates, rectal swabs and faeces of ITU patients and stored on nutrient agar slopes in darkness at room temperature. These were termed the SPEAR isolates. The aim of the present work was to investigate the cefotaxime resistance of these organisms. Of the 200 isolates in the collection 17 were excluded at the outset: 13 had been submitted from a patient in another ITU, two isolates had not survived and the identification data for another two was erroneous. Of the 183 remaining, ten had been recorded as resistant to tobramycin but sensitive to cefotaxime. Cefotaxime sensitivity was confirmed in eight of these isolates which were therefore removed from the study. As two of the ten tobramycin-resistant isolates (although sensitive *in vitro* to cefotaxime) displayed features suggestive of ESBL production they were included for full investigation along with the 173 cefotaxime-resistant strains. Therefore 175 isolates were investigated in this study.

Species identification was carried out using the API 20 E and 20 NE systems (bioMérieux, Marcy-l'Etoile, France). Nine isolates - 13, 15, 18, 28, 29, 30, 31, 32 and 95 - grew optimally at 30°C rather than at 37°C. Disk diffusion tests, minimum inhibitory concentrations (MICs) and transconjugation experiments were carried out at 30°C on these organisms.

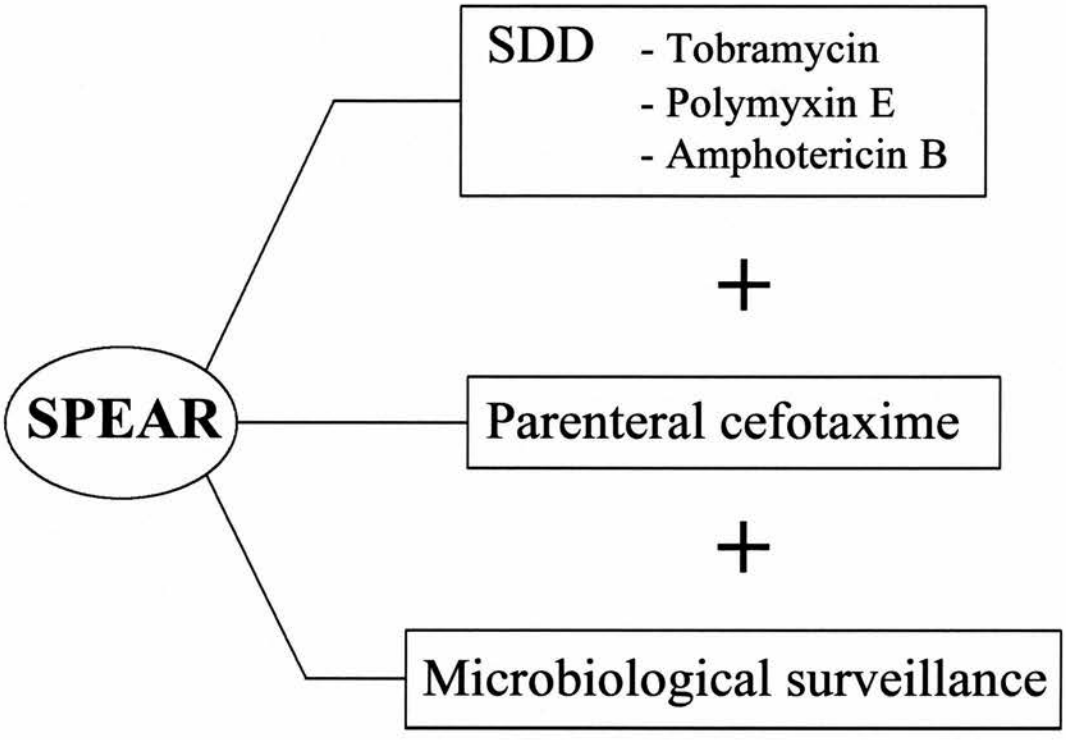

Figure 4 The three components of SPEAR.

2.1.2 The patients

The SPEAR isolates were collected from 92 patients aged from 17 to 83 years (Appendix I) out of a total number of 969 patients admitted to a general medical/surgical five-bedded ITU (Figure 5) over a period of 2 years and 10 months. The patients comprised 49 male patients (aged from 19 to 78 years) and 43 female patients (aged from 17 to 83 years). The length of time spent in ITU ranged from 12 hours to 36 days but the mean duration of stay was 9.8 days. Admission to ITU was for medical reasons in 26 patients and for surgical reasons in 66 patients. Cefotaxime therapy before admission to ITU was recorded for only five patients (3, 13, 14, 31, and 51).

2.2 Antimicrobial susceptibility testing

2.2.1 Antibiotics

The following antibiotic disks were purchased from Oxoid (Basingstoke, UK): amoxycillin 20 μg and clavulanic acid 10 μg "co-amoxyclav" (AMC 30); ampicillin 10 μg (AMP 10); azlocillin 75 μg (AZL 75); aztreonam 30 μg (ATM 30); carbenicillin 100 μg (CAR 100); cefotaxime 30 μg (CTX 30); cefoxitin 30 μg (FOX 30); cephalexin 30 μg (CL 30); ceftazidime 30μg (CAZ 30); ciprofloxacin 5 μg (CIP 5); erythromycin 15 μg (E 15); gentamicin 10 μg (CN 10); imipenem 10μg (IPM 10); mecillinam 25 μg (MEL 25); meropenem 10 μg (MEM 10); oxacillin 1 μg (OX 1); penicillin G 10 units (P 10); piperacillin 100 μg (PRL 75); piperacillin 100 μg and tazobactam 10 μg "tazocin" (TZP 110); ticarcillin 75 μg (TIC 75); ticarcillin 75 μg and clavulanic acid 10 μg "timentin" (TIM 85); trimethoprim 5 μg (W 5). Temocillin 30 μg (TEM 30) disks were obtained from Mast Laboratories Ltd. (Bootle, Merseyside, UK).

Figure 5 The general medical/surgical ITU where the isolates were collected from patients undergoing SPEAR.

Adatabs (ampicillin, cefotaxime, ceftazidime, and nalidixic acid) for agar dilution susceptibility testing were obtained from Mast Laboratories Ltd. (Bootle, Merseyside, UK) and antibiotic powders were obtained as follows: rifampicin (R 3501, Sigma Chemical Co., Poole, Dorset, UK); Rifampicin BP (Marrion Merrell Dow Ltd, Uxbridge, Middlesex, UK).

Etest® strips (AB Biodisks, Solna, Sweden) for the detection of ESBLs were purchased from Cambridge Diagnostics Services Ltd, Cambridge, England).

2.2.2 Disk diffusion tests

Disk diffusion tests were carried out according to National Committee for Clinical Laboratory Standards (NCCLS) criteria [192]. Organisms were grown overnight on nutrient agar (CM271, Oxoid, UK) and checked for purity. The inoculum was prepared by touching the tops of four separate colonies [193] of each isolate with a sterile disposable loop (Technical Service Consultants Limited, Heywood, Lancashire) and suspending in 0.85% sterile saline. The inoculum density was adjusted immediately to match a 0.5 McFarland barium sulphate turbidity standard and a sterile swab was dipped into the inoculum suspension. The swab was rotated several times pressing on the inside wall above the fluid level to remove excess inoculum. The dried surface of a Mueller-Hinton agar (CM337, Oxoid, UK) plate was inoculated by streaking the swab over the entire agar surface three times while rotating the plate to ensure an even distribution of inoculum (and a confluent or almost confluent lawn of growth after incubation). The appropriate drug-impregnated disks were then placed on the surface of the agar either with sterile forceps or with disk dispensers (Oxoid, UK) and the plates incubated for 18 hours at 37°C or 30°C depending on the optimal growth temperature of the organism. Zones of inhibition, including the 6 mm disk diameter, were measured to the nearest mm using a ruler and recorded. The sizes of the zones of inhibition were interpreted according to NCCLS guidelines [192].

2.2.3 Determination of cefotaxime MICs for SPEAR isolates

A standard agar dilution antimicrobial susceptibility test was used [194]. Doubling dilutions of cefotaxime (Adatabs, Mast Laboratories Ltd., Bootle, Merseyside) ranging from 32 mg/L to 0.5 mg/L were prepared in molten Mueller-Hinton agar (CM337, Oxoid, UK) at a temperature of approximately 50°C. Aliquots of each dilution were poured into 85 mm diameter sterile plastic petri dishes (Sterilin, UK), to a uniform depth of 3-4 mm on a level horizontal surface and the agar allowed to cool and solidify at room temperature. Plates containing Mueller-Hinton agar without antibiotic were prepared at the same time to act as growth medium controls. The plates were used immediately or stored at 4°C and used within 72 hr.

The purity of each isolate was verified by plating out for single colonies on nutrient agar (CM271, Oxoid, UK) plates which were incubated aerobically overnight at 37°C or 30°C according to the optimal growth temperature of the organism. The inoculum was prepared by touching the tops of four separate colonies [193] of each isolate and incubating in 10 mL Isosensitest broth (CM473, Oxoid, UK) overnight at the appropriate temperature. Using a multipoint (19-pin) hand inoculator and teflon inoculum pot (Mast Diagnostics, Merseyside) the inoculum was applied in each case at about 3×10^4 cfu per inoculum spot to each of the cefotaxime dilutions in the prepared plates which were incubated overnight at the appropriate temperature. An identical preparation of a strain of *Escherichia coli*, National Collection of Type Cultures (NCTC) accession number 101418 (Public Health Laboratory Service, Colindale Avenue, London) was used to test the consistency of the results with each set of test isolates. The next day the MIC of cefotaxime for each isolate was read as the lowest concentration of the antibiotic which had inhibited its growth [194] (Appendices II and IV).

Isolates 133 and 140 (*Klebsiella pneumoniae*) were further tested by the methods described above in a series of cefotaxime dilutions ranging from 2 mg/L to 0.0125 mg/L.

2.2.4 Etest® for ESBL detection

The Etest® (AB Biodisk, Solna, Sweden) for the detection of ESBLs was carried out on all the SPEAR isolates. The Etest is based on the concepts of both dilution and diffusion tests. As Etest MIC values are directly proportional to NCCLS dilution results, NCCLS breakpoints are appropriate for susceptibility categorization (AB Biodisk, Solna, Sweden). The Etest for ESBL detection consists of a thin, inert and non-porous plastic strip carrying, at opposite ends, two predefined exponential gradients of dried and stabilized antibiotics - TZ (ceftazidime 0.5-32 mg/L) and TZL (ceftazidime 0.064-4 mg/L with clavulanic acid 4 mg/L). When the Etest strip is applied to an inoculated agar plate the antibiotics are immediately released from the carrier surface into the agar matrix. After incubation symmetrical inhibition ellipses are seen unless the organism is resistant to the concentrations of antibiotics used.

Each SPEAR isolate was plated out on nutrient agar (CM271, Oxoid, UK). After appropriate incubation an inoculum suspension having a turbidity equivalent to a 0.5 McFarland standard was prepared from each isolate in a test tube containing 0.85% sterile saline [192]. A sterile swab was dipped into each inoculum suspension and excess fluid removed by rotating and pressing the swab firmly against the inside wall of the test tube. The swab was then used to inoculate the entire surface of a Mueller-Hinton agar plate (CM337, Oxoid, UK) with care to ensure an even distribution of inoculum. The plates were allowed to sit for approximately ten minutes so that their surfaces were completely dry before applying the Etest strips. *Escherichia coli* NCTC 10418 was used as an ESBL-negative control and an SHV-2-producing strain of *Escherichia coli* (kindly provided by Professor SGB Amyes)

used as an ESBL-positive control. After overnight incubation at 37°C or 30°C (depending on the optimum growth temperature of the isolate) the MIC values of ceftazidime and ceftazidime with clavulanic acid for each isolate were read at the point of intersection between the appropriate inhibition ellipse edge and the Etest strip.

2.2.5 Ceftazidime MICs for SPEAR isolates

These were determined as an integral part of the Etest® for ESBL detection (AB Biodisk, Solna, Sweden) as described above.

2.3 Plasmid analysis

2.3.1 Introduction

Most methods for detecting plasmids are variations on the theme of harvesting bacterial cells, lysing the cells, centrifuging the lysate to remove cell debris, purifying the resultant DNA and subjecting it to electrophoresis on agarose gels. Following electrophoresis the gels are stained with ethidium bromide which intercalates between adjacent base pairs in DNA and fluoresces under ultraviolet light. DNA can be seen as bright orange bands in the gels with the use of a transilluminator.

In plasmid preparations it is necessary to separate the plasmid DNA from the large amount of bacterial chromosomal DNA which is also present in the cells. The methods are based on the physical differences between plasmid DNA and chromosomal DNA, the most obvious of which is size. The largest plasmids found in bacteria are only about eight per cent of the size of the *Escherichia coli* chromosome (4700 kb) and most are smaller. Techniques which separate small DNA molecules from large ones can purify plasmid DNA to the extent required by screening.

The usual stage at which to attempt separation on the basis of size is during preparation of the cell extract. If the cells are lysed under very carefully controlled conditions then only a minimal amount of chromosomal DNA breakage will occur. The bacterial cell is enclosed in a cytoplasmic membrane and surrounded by a rigid cell wall. Gram-negative bacteria are enveloped by an outer membrane. These barriers have to be disrupted to release the cell components. Chemical rather than physical methods are most commonly used in DNA preparation. The chemicals that are used depend on the species of bacterium involved. Weakening of the cell wall is usually brought about by ethylenediaminetetraacetic acid (EDTA), lysozyme, or a combination of both. EDTA removes divalent cations (magnesium Mg^{2+} and calcium Ca^{2+}) which help stabilize the membrane by combining with negative charges of the phospholipids [195 {p63}] and are essential for preserving the overall structure of the cell envelope, producing a non-specific increase in cellular permeability [196,197]. EDTA, by chelating metal ions, also inhibits cation-dependent nucleases which could degrade DNA [198]. Lysozyme is a protein which breaks the ß 1,4-glycosidic bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid in peptidoglycan.

The outer membrane of Gram-negative bacteria is disrupted by EDTA and this allows lysozyme to degrade the peptidoglycan layer of the cell wall so that sphaeroplasts are formed. In order to expedite a more controlled, gentle lysis these reactions may be carried out in the presence of a sufficient concentration of sucrose or other sugar to prevent immediate osmotic lysis of the sphaeroplasts [198]. Under some conditions weakening of the cell wall with lysozyme or EDTA is sufficient to cause the bacterial cells to lyse, but usually a detergent such as sodium dodecyl sulphate (SDS) or Triton X-100 is also added. Detergent molecules disrupt membranes by intercalating into phospholipid bilayers and solubilizing lipids and proteins [199 {p582}]. Ionic detergents such as SDS contain a charged polar head. They bind to the hydrophobic regions of proteins. They also alter the conformation

of the hydrophilic regions and disrupt non-covalent bonds such as ionic and hydrogen bonds. At high concentrations SDS completely denatures proteins.

Nonionic detergents such as Triton X-100 do not have a charged group. Usually they solubilize biological membranes by forming mixed micelles of detergent, phospholipid and integral membrane proteins. They generally do not denature proteins [199 {p583}] and are thought to promote a more gentle lysis [200]. However some bacteria are not lysed by them.

At this stage the bacterial cell extract contains chromosomal DNA, plasmid DNA, protein and ribonucleic acid (RNA). If the cells have been lysed under carefully controlled conditions then only a small amount of chromosomal DNA breakage will have occurred. The resulting chromosomal DNA fragments are still much larger than the plasmids and are removed with the cell debris by centrifugation, a clearing spin, of the crude cell extracts to leave a cleared lysate consisting mainly of plasmid DNA [201 {p32}]. This process is aided by the fact that the bacterial chromosome is physically attached to the cell envelope and fragments are likely to sediment with the cell debris if these attachments are not broken. A cleared lysate does, however, invariably retain some chromosomal DNA. This is the reason why size fractionation on its own is insufficient if pure plasmid DNA is required. Furthermore, if the plasmids are themselves large molecules then they may also sediment with the cell debris.

In addition to size, plasmid and chromosomal DNA differ in conformation. This refers to the overall spatial configuration of the molecule, with the two simplest conformations being linear and circular. The bacterial chromosome is circular, but during preparation of the cell extract the chromosome will always be broken to give linear fragments. It is simplistic to say that plasmids have a circular conformation, because double-stranded DNA circles can in fact take up one of two quite distinct configurations. Most plasmids exist in the cell as supercoiled molecules. Supercoiling occurs because the double helix of the plasmid DNA is partially

unwound by DNA gyrase during the plasmid replication process. The supercoiled configuration can be maintained only if both polynucleotide strands are intact and is known as covalently closed circular (CCC) DNA. If one of the polynucleotide strands is broken, then the double helix will revert to its normal relaxed state and the plasmid will take on the alternative, open circular (OC), conformation.

Supercoiling is exploited in plasmid preparations because supercoiled molecules can be separated from non-supercoiled DNA in crude cell extracts or cleared lysates by such rapid methods as boiling or alkaline denaturation [202 {p1.25-1.30}] or the very time-consuming, labour-intensive and expensive process of caesium chloride-ethidium bromide density gradient centrifugation [202 {p1.42-1.46}]. The plasmid DNA resulting from the last method is virtually pure whereas the rapid preparations are invariably contaminated to a greater or lesser extent with chromosomal DNA. However these rapid preparations are often in practice adequate for analysis with restriction enzymes, for transformation and even in many cases for cloning [122 {p14},200] without resorting to caesium chloride-ethidium bromide density gradient centrifugation [202 {p1.23-1.24}].

Boiling or treatment with alkali disrupts base pairing, causing the linear chromosomal DNA to denature. However the strands of CCC plasmid DNA are unable to separate from one another because they are topologically intertwined. When conditions are returned to normal, provided the exposure to heat or alkali has not been prolonged, the strands of the plasmid DNA rapidly "fall into perfect register" and completely native superhelical molecules are re-formed [202 {p1.22}].

An additional advantage of the alkaline denaturation procedure is that under certain circumstances most of the protein and RNA will also become insoluble and be removed by the centrifugation step [201 {p33}]. In the cases of the other rapid procedures phenol or phenol:chloroform extraction and ribonuclease treatment are needed to remove protein and RNA [200,201 {p27}]. Following any of the rapid

preparations the plasmid DNA can be concentrated finally by precipitation, usually with ethanol [203 {pE.10 - E.14}]

The choice of method for preparation of plasmid DNA is determined largely by the aims of the investigator and depends on how pure the plasmid DNA has to be and the quantity required. If the intention is to digest the DNA subsequently with restriction enzymes or more especially to use it in cloning experiments then it is essential to purify the DNA from clinical isolates.

2.3.1.2 Plasmid trapping

Cell density is the major parameter altered when preparing plasmid DNA from different genera (DJ Platt, personal communication). What exactly is the optimum cell density? The process has not received much attention possibly because many molecular biologists are working with small vector plasmids in *Escherichia coli*. In the process of lysis and centrifugation, lysis releases cell contents including plasmid and chromosomal DNA and the lysate becomes viscous. Centrifugation pellets cell debris, including the majority of the chromosomal DNA except small linear shear-fragments. However as this material travels to the bottom of the tube it can trap plasmid DNA thereby reducing the yield. If this results in a final concentration of plasmid DNA below the critical threshold for ethidium bromide detection, then false negative results will be obtained.

Plasmid trapping may differ with genus because the properties of chromosomal DNA differ (DJ Platt, personal communication). The theory is that the genomes of *Klebsiella*, *Enterobacter* and *Serratia* species are larger than that of *Escherichia coli*. Whereas *Escherichia coli* would continue to give positive results at high cell densities, strains of *Klebsiella*, *Enterobacter* and *Serratia* species would suffer from excessive plasmid trapping by their large chromosomes at higher cell densities and this would reduce the effective range of cell densities for these genera. A reduced

cell density could be used in the preparation of the lysates but presumably there would be a critical mass of cells below which plasmids would not be detected.

Are the chromosomes of *Klebsiella*, *Serratia* and *Enterobacter* species larger than that of *Escherichia coli* ? Prior to 1970 the genome size of bacterial DNA was known only for a very few species [204]. With the application of the technique of renaturation of single-stranded DNA substantial data became available [205]. The sizes quoted for the genome of *Escherichia coli* ranged from 2200 to 2980 megadaltons (MDa) [204,206,207]; for *Klebsiella* species 2360 to 2580 MDa [204,206]; for *Serratia marcescens* 5020 to 5560 MDa [204] and for *Pseudomonas* species 2530 to 6960 MDa [204,206]. A reference for genome size in *Enterobacter* species was not found [204]. Modern sequencing methods [208] are now in progress for the determination of bacterial genome sizes: that of *Escherichia coli* is reported as 4600 kb (2921 MDa). Work on *Klebsiella pneumoniae* strain M6H 78578 is in progress. There is no information yet on the genome sizes of *Enterobacter* or *Serratia* species. The genome size of *Pseudomonas aeruginosa* strain PAO1 is reported as 5900 kb (3746 MDa).

Another way to approach the problem is not to reduce the cell density but to emulsify the cells in electrophoresis buffer with additional EDTA, which has mild detergent activities and prevents nuclease activity. The overall effect may be a gentler lysis of the cells and protection of the supercoiling of DNA with less shearing of the chromosome. An additional factor in some genera is the fragility of chromosomal DNA. In lower cell densities of *Salmonella*, large plasmids can be detected readily but plasmids < 30 kb are obscured because chromosomal shear-fragments occupy a more extensive region of the track than is usual. This can be overcome by using a higher cell density. It seems that in this situation a higher concentration of chromosomal DNA protects against shear without significant reduction in plasmid yield by entrapment (DJ Platt, personal communication). The

role of chromosomal trapping of plasmids may vary also with the size of the plasmid: the larger the plasmid the more easily it is trapped.

2.3.2 Preparation of plasmid DNA

One of the aims of the present study was to determine whether the resistance of any of the SPEAR isolates to cefotaxime was plasmid-mediated. It was necessary to find out first whether the isolates actually carried plasmids and then to carry out transconjugation experiments to determine whether the cefotaxime resistance could be transferred to a recipient organism by transfer of a plasmid.

The method chosen for this work was based on that described by Platt and colleagues [209]. It had been designed to generate plasmid profiles from large numbers of clinical isolates with speed and flexibility and to be applicable, with minimal modifications, to diverse bacterial genera.

The purity of each isolate was checked by plating out for single colonies on nutrient agar (CM271, Oxoid, UK) and incubating overnight at 37°C or 30°C. Using a sterile disposable plastic loop (Technical Service Consultants Limited, Heywood, Lancashire) 3 loopfuls of bacterial culture were transferred to a 1·5 mL eppendorf tube (Treff, Scotlab, Paisley, Scotland) containing 300 µL of Tris-borate-EDTA (TBE) electrophoresis buffer pH 8·3 comprising Trizma, Tris(hydroxymethyl)aminomethane base 89 mM, boric acid 89 mM, and EDTA 1.25 mM (all Sigma Chemical Co., Poole, Dorset, UK). The pH of the TBE buffer was measured using a Tris-compatible electrode [210 {p5-6}]. In certain circumstances 50 µL EDTA (50 mM) were added to the 300 µL aliquot of TBE electrophoresis buffer at this stage (see above). After thoroughly emulsifying the bacterial cells in the buffer using a sterile wooden swab stick, 200 µL SDS (Sigma Chemical Co., Poole, Dorset, UK) 10% weight/volume solution in TBE buffer, were added to the eppendorf tube which was inverted two or three times very gently in order to mix the contents and then incubated in a water bath at 50°C for five to ten

minutes to aid bacterial cell lysis. On removal from the water bath the eppendorf tube was centrifuged for 15 minutes at 11,600 *g* (MSE Micro-Centaur centrifuge, MSE, Loughborough, Leicestershire, UK). The pellet of bacterial and chromosomal debris was removed using a micropipette, leaving behind the supernatant containing the plasmid DNA, i.e. the cleared lysate [201 {p32}].

2.3.2.1 Observations

The crucial step was the initial emulsification of the harvested bacteria in TBE buffer and this seemed to depend greatly on the slime content of the cells. When this was excessive fewer cells could be emulsified and the concentration of DNA must inevitably have been smaller. Attempts to emulsify too high a cell density resulted in minimal or no supernatant (i.e. lysate) after the centrifugation stage, i.e. everything was entrapped by slime. This happened more often with isolates of *Acinetobacter* species and *Pseudomonas* species. Harvesting the bacteria in nutrient broth rather than solid media, followed by several washes either in nutrient broth or in TBE buffer followed by centrifugation did not improve emulsification in several strains of *Acinetobacter* and *Pseudomonas* species. Moreover, the practice of using TBE buffer with additional EDTA as described above to minimise plasmid trapping was not appropriate for certain mucoid isolates which emulsified even less easily in this solution. In the case of very mucoid isolates it was helpful to use cultures after approximately twelve hours of incubation when the slime production was less.

A modification of an alkaline extraction technique [211] was tried out only once in conjunction with the method of Platt and colleagues [209] on the *Escherichia coli* strain 39R861 which carried four of the reference plasmids (bacterial strain and plasmids to be described below). RNAase treatment of the resulting lysates was not required, otherwise the results were not as good as those achieved using the method of Platt and colleagues [209] on its own.

2.3.2.2 Reference plasmids

Six reference plasmids were used routinely for molecular weight determinations. The largest of these, RTS 1 [212], contained in an *Escherichia coli* K12 host J53-1 was kindly provided by DJ Platt, Department of Bacteriology, Glasgow Royal Infirmary as a reference plasmid of 120 MDa. In transconjugation experiments carried out in *Escherichia coli* hosts Ishihara and colleagues [213] found that RTS 1 gave rise to two kinds of deletion mutants with molecular weights of approximately 120 MDa and 100 MDa in co-sedimentation studies with known plasmids using alkaline sucrose density centrifugation techniques. They reported the molecular weight of the "original RTS 1" plasmid as 140 MDa [213]. However the RTS 1 plasmid, part of the Datta Collection [214 {p1}], had been donated to Dr Platt by Professor Datta as a plasmid of molecular weight 120 MDa "in her hands" (DJ Platt, personal communication). Platt and colleagues [209] used RTS 1 as a reference plasmid of molecular weight 120 MDa (174 kb) multiplying by a factor of 1·45 to convert from MDa to kb. Throughout the present work the more recently recommended conversion factor of 1.575 [203 {pC.3}] has been used. The molecular weight of the RTS 1 reference plasmid was therefore designated 189 kb.

Strain 39R861 NCTC 50192, an *Escherichia coli* K12 construct (kindly provided by DJ Platt), was used as a molecular weight standard containing four reference plasmids of molecular weights 98, 42, 23·9 and 4·6 MDa [215] i.e. 154·35 kb, 66·15 kb, 37·64 kb and 7·25 kb respectively.

The sixth and smallest reference plasmid was pUC9, molecular weight 2·68 kb, in host strain *Escherichia coli* DS 941 donated by Professor D Sherratt, Microbiology Unit, Department of Biochemistry, University of Oxford.

Cell lysates of the three bacterial host strains containing the reference plasmids were prepared exactly as those of the test isolates. These reference plasmids were included in each run in order to check the technical acceptability of the system and

to allow sizing of the test plasmids by comparing their distance of migration with that of the reference plasmids.

DNA extracts of a very large plasmid pMG5 (491 kb), contained in the host strain *Pseudomonas aeruginosa* PU21 (NCTC 50534), were also prepared as described above and subjected to electrophoresis in order to determine whether the system was capable of detecting such a large plasmid.

2.3.2.3 Preparation of cleared lysates for loading on to gels

A 45 µL volume of each lysate was transferred to a sterile eppendorf tube and ribonucleic acid (RNA) was removed using Ribonuclease A (RNAase, Sigma Chemical Company, UK), 1 µL of a 1mg/mL solution in deionized water per 10 µL of lysate. Prior to storage stock solutions (10 mg/mL) of RNAase were boiled for 15 minutes to destroy any possible DNAase activity. Loading buffer consisting of glycerol (Sigma Chemical Company, UK) 30% in deionized water and bromophenol blue (Sigma Chemical Company, UK) 0.25% was added in volumetric ratio of 1 to 6 [202 {p6.12}] with the lysate to ensure that the preparation did not float out of the well and to enable the sample to be visualized during electrophoresis. The total sample volume routinely loaded was 45 µL.

2.3.3 Agarose Gel Electrophoresis

2.3.3.1 Introduction

Electrophoresis through agarose or polyacrylamide gels is the standard method used to separate, identify and purify nucleic acids [202 {p6.2}]. DNA is negatively charged by virtue of its phosphate groups. Electrophoresis is the procedure by which charged molecules are allowed to migrate in an electric field [195 {p189}], their rate of migration or mobility depending on the strength of the field, on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. The gel is a complex network of fibrils which acts as a molecular sieve and its pore size can be

controlled by the way in which the gel is prepared. The nucleic acid molecules migrate through the pores at rates dependent on their molecular weight and molecular shape. Small or compact molecules migrate more rapidly than large or loose molecules which are impeded to a greater extent by the gel matrix. DNA can exist in various conformations, CCC, OC and linear forms and although this does not affect charge, it does affect molecular sieving.

Agarose, a highly purified polysaccharide extracted from seaweed, is a linear polymer whose basic structure consists of alternating D-galactose and 3,6-anhydro L-galactose molecules [202 {p6.3}]. Agarose, which comes in powder form dissolves when added to boiling liquid. It remains in a liquid state until the temperature is lowered to about 40°C, at which point it gels. The gel is stable and will not dissolve again until the temperature is raised back to about 100°C. Its pore size may be predetermined by adjusting the concentration of agarose in the gel: the higher the concentration, the smaller the pore size. Therefore agarose gels can be poured in a variety of shapes, sizes and porosities and can be run in a variety of configurations.

The most commonly used configuration is the horizontal slab gel [202 {p6.8}] which is usually poured on a glass plate or plastic tray which can be installed on a platform in the electrophoresis tank. Electrophoresis is carried out with the gel submerged just beneath the surface of the buffer i.e. horizontal submarine gel electrophoresis. The resistance of the gel to the passage of electric current is almost the same as that of the buffer and so a considerable fraction of the applied current passes along the length of the gel. Grinsted and Bennett [216] recommend horizontal rather than vertical gel electrophoresis because horizontal gels are much easier to prepare, use and process afterwards. However one advantage of vertical gels is that the wells can be deeper and therefore that larger samples can be run.

Agarose gels are cast by melting the agarose in an appropriate buffer until a clear, transparent solution is achieved. The melted solution is poured into a mould or gel

tray and allowed to harden. On hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. When an electric field is applied across the gel, DNA, which is negatively charged, migrates towards the anode [202 {p6.3}]. After a defined period of time of migration the locations of the DNA molecules in the gel are assessed by making them fluorescent and observing the gel with ultraviolet radiation.

Plasmids vary greatly in size from about 1 kb to greater than 400 kb [217]. The problem is that natural isolates can contain multiple plasmids, both large and small, which must be displayed on the same gel to give a plasmid profile [200].

The use of agarose gel electrophoresis for the detection and preliminary characterization of CCC plasmid DNA in clinical isolates and laboratory strains of Gram-negative bacteria was first reported in 1976 [218]. Prior to this Aaij and Borst [219] had reported that the migration rates of purified bacteriophage and mitochondrial CCC DNAs ranging from 3·4 x 10^6 to 10 x 10^6 daltons, i.e. 5 to 16 kb, were related inversely to the logarithm of their mass in 0·6% agarose gels. Subsequently agarose gel electrophoresis was employed widely in the analysis of restriction-endonuclease-generated fragments of plasmid and viral DNA [218]. However the migration properties of higher molecular weight CCC DNA had been overlooked. Plasmids in a fresh preparation usually consist of double-stranded DNA and are generally isolated as circular molecules in which each of the single strands is a covalently closed circle [200]. A small amount of the OC form may be present which increases as the preparation ages and sometimes even the linear form eventually appears [200].

Meyers and colleagues [218] found agarose gel electrophoresis to be suitable for the detection and estimation of plasmid DNA of molecular weight ranging from 0.6 x 10^6 to 95 x 10^6 daltons in partially purified whole-cell lysates. Expressed in kb [220] this is a size range from approximately 0·9 to 150 kb, as 10^6 daltons are equivalent to 1·575 kb [203 {pC.3}]. Meyers and colleagues [218] found that the

migrations of these CCC plasmid DNA species were related inversely to their molecular weights i.e. the larger the molecular weight the slower the migration. Willshaw and colleagues [221] further investigated the problems encountered in using agarose gel electrophoresis in the analysis of wild enterobacterial strains. Using 0.75% agarose gels they obtained a reliable estimate of molecular sizes over a wide range, 3 x 10^6 to 80 x 10^6 daltons (5-126 kb) for which molecular weight and relative mobility were linearly related. Gels were calibrated using a plot of the logarithm of relative migration of the DNA through the gel versus the logarithm of plasmid molecular weight. Within the range quoted above, plasmid sizes determined by electrophoresis were within 10% of the values determined by electron microscopy and physical measurements. For large plasmids up to 144 x 10^6 daltons (227 kb) the linear relationship was no longer valid and the resolution of plasmid species was reduced. With 1% agarose plasmid mobility was a linear function of molecular size from 3 x 10^6 daltons to 50 x 10^6 daltons (5 to 79 kb) and these conditions were not used for the molecular weight determination of large plasmids. Molecular weights in excess of 80 x 10^6 daltons (127 kb) could be determined on 0.65% gels but at this agarose concentration the mobilities of plasmids smaller than 10 x 10^6 daltons (16 kb) were less than would be predicted from a linear relationship between molecular weight and relative mobility [221].

2.3.3.2 Observations

Meyers and colleagues [218] obtained partially purified plasmid DNA by ethanol precipitation of phenol-treated cleared lysates. Willshaw and colleagues [221] used "crude ethanol-precipitated DNA". Both Meyers and colleagues [218] and Willshaw and colleagues [221] used vertical agarose gels of 15 cm length. Sample volumes were 5, 10 or 25 µL [218] and 2-100 µL [221].

In the present work partial purification of plasmid DNA was carried out as described by Platt and colleagues [209] who used cleared lysates without further

purification (i.e. without phenol treatment and ethanol precipitation). They used large volumes (100 μL) of these "crude" lysates on vertical gels of 15 cm length whereas in the present work commercial horizontal gel electrophoresis apparatus was used with necessarily smaller (45 μL) volumes of lysates.

These observations raised a number of questions. Would the smaller volumes of lysates reduce the sensitivity of horizontal gels? Would horizontal gels have to be longer than vertical gels to compensate for "lack of gravity" and to allow time for large plasmids to enter the gels before smaller plasmids had migrated off the ends? Would large plasmids even enter horizontal gels? At what voltage should the gels be run? Would a higher voltage be needed to drive large plasmids into horizontal gels?

When choosing electrophoresis conditions a balance has to be struck between sharpness and separation [222]. In general, large DNA fragments are best resolved by subjecting gels to electrophoresis for long times at low voltage gradients. According to Sambrook and colleagues [202 {p6.6}] agarose gels should be run at no more than 5 V/cm to obtain maximum resolution of DNA fragments greater than 2 kb in size but the authors were referring to linear DNA fragments. According to Sealey and Southern [222] smaller fragments diffuse faster and therefore band sharpness is increased by using high voltage gradients for electrophoresis. These authors describe analogous situations with regard to gel length and gel concentration. The further that fragments migrate the greater is the separation between them. However increasing the migration distance requires either longer times or higher voltage gradients which may lead to increased diffusion of small fragments or less separation of large fragments respectively. In general separation is better on gels of higher concentration for equivalent distances migrated. However large DNA fragments move so slowly in concentrated gels that lower concentration gels must be used [222]. In order to encompass some of these variables, three horizontal electrophoresis systems of different dimensions were used in the present work to screen the SPEAR isolates.

2.3.3.3 Materials and Methods

Three horizontal electrophoresis systems of different sizes were used (Figure 6): the GNA-100 (Pharmacia Molecular Biology, Uppsala, Sweden) mini-gel, the Horizon™ 11•14 medium-size (11 x 14 cm) gel and the Horizon™ 20•25 large (20 x 25 cm) gel (both Gibco BRL, Paisley, Scotland, UK). Each SPEAR isolate was examined at least twice: once on a medium-length (14 cm) and once on a long gel (25 cm) and often on multiple gels. Transconjugants from the control broth-mating systems were examined on the mini-gel system and those from the additional transconjugation studies were examined on both the mini- and medium-gel systems.

Ultra-pure electrophoresis grade agarose (Gibco BRL, Paisley, Scotland) was weighed and transferred to a glass beaker. An appropriate volume of TBE buffer, as described above in the preparation of plasmids, was added to give an agarose concentration of 0•7%. The beaker was placed on a tripod stand over a Bunsen burner and heated until the agarose dissolved in the boiling liquid and the solution appeared clear. The mixture was swirled continuously around the container during heating to prevent scorching. The molten agarose was then placed in a water bath at 50°C. Gels were prepared by pouring the molten agarose, cooled to 50°C, to a depth of 5-6 mm on to the bubble-levelled ultraviolet light translucent trays of the GNA-100 (Pharmacia Molecular Biology, Uppsala, Sweden) horizontal mini-, medium or large gel electrophoresis systems.

Figure 6 The power supply, mini-, medium and large gel tanks used in agarose gel electrophoresis.

Wells were formed in each gel according to the apparatus being used by the positioning of one of a variety of Noryl® plastic (Pharmacia Molecular Biology, Uppsala, Sweden) or Delrin® (Gibco BRL, Paisley, Scotland, UK) combs of different sizes in the molten agarose and removing them when the agarose was completely set (approximately 30-45 minutes at room temperature) by which time the gel had assumed a slight "bloom" or opacity. Removal of the comb without tearing the bottom of the wells was facilitated each time by very gently wiggling the comb to free the teeth from the gel, slightly lifting up one side of the comb, then the other.

TBE buffer (described above in the preparation of plasmid DNA) was used as the running electrophoresis buffer. The same batch of TBE buffer was always used in both the electrophoresis tank and the gel to prevent small differences in ionic strength or pH creating fronts in the gel which might affect the mobility of DNA fragments [202 {p6.9}]. TBE buffer was poured into the gel tank until the gel was submerged to a depth of no more than 1-2 mm to prevent drying of the gel and to ensure an even voltage gradient across the gel bed. Submerging to a depth greater than 2 mm is unnecessary and increases electrical current and heat. The prepared lysates containing loading buffer were each carefully loaded, under the buffer, into the wells using a micropipette and disposable 200 μL sterile plastic tips.

Each electrophoresis apparatus was fitted with a safety lid which could not be removed while electrical current was flowing through the system. In each case power was provided by a constant voltage Gene Power Supply (Figure 6), GPS 200/400 (Pharmacia LKB Biotechnology, Uppsala, Sweden). Routine running parameters for each electrophoresis system were 100 V for 2.5 hours (GNA-100), 100 V for 3.5 hours (Horizon™ 11•14) and 150 V for 6.5 hours (Horizon™ 20•25). All gels were run at room temperature.

Lambda DNA/*Hin*d III fragments (Gibco BRL, Paisley, Scotland) were included in many runs only because of familiarity with their optimum appearance in a

technically satisfactory run. It must be emphasized that the digest markers in this preparation consist of linear DNA and as such were NOT appropriate for comparative sizing of plasmid DNA which is mainly supercoiled.

2.3.4 Analysis of gels after electrophoresis

2.3.4.1 Introduction

The most convenient way of visualizing DNA in agarose gels is staining with the fluorescent dye ethidium bromide [223] which contains a planar group which intercalates between the stacked bases of DNA. The fixed position of this group and its close proximity to the bases cause dye bound to DNA to display an increased fluorescent yield compared to that of dye in free solution. Ultraviolet radiation at 254 nm is absorbed by the DNA and transmitted to the dye whereas radiation at 302 nm and 366 nm is absorbed by the bound dye itself. In both cases the energy is re-emitted at 590 nm in the red-orange region of the visible spectrum. Small amounts of DNA can be detected in the presence of free ethidium bromide in the gel because the fluorescent yield of ethidium bromide:DNA complexes is much greater than that of unbound dye [202 {p6.15}].

2.3.4.2 Materials and methods

On completion of the run, the gel was removed from the tank and stained with ethidium bromide (Sigma Chemical Co., Poole, Dorset, UK) at a concentration of 0.5 mg/L in TBE electrophoresis buffer for 45 min. The gel was then destained for 20 min in changes of TBE buffer and deionized water in order to reduce excessive background fluorescence of the gels and improve photographic appearances. Prolonged destaining can cause loss of DNA-bound dye.

In order to analyse the pattern obtained and to keep a permanent record, photography of the gels was carried out in a darkroom using a Polaroid MP-4 Land Camera (Polaroid Ltd, Ashley Road, St Albans, Hertfordshire,UK) and Polaroid Type 667 black and white film ISO 3000 (Sigma Chemical Co, Poole, Dorset, UK)

while the gels were transilluminated with ultraviolet light provided by either a short-wave (254 nm) or medium-wave (302 nm) transilluminator (UV Products Ltd, Science Park, Milton Road, Cambridge, UK) depending on the availability of access to equipment. Most of the photographs were taken using the medium-wave (302 nm) transilluminator. The fluorescent yield of ethidium bromide:DNA complexes is considerably greater at 302 nm than at 366 nm and slightly less than at short-wavelength (254 nm) light. However the amount of nicking of the DNA is much less at 302 nm than at 254 nm [202 {p6.19}]. Therefore for general use illumination at 302 nm is preferred [222]. Photographs were taken through filters: a Kodak 2B Wratten ultraviolet-blocking filter (Sigma Chemical Co., Poole, Dorset, UK) was used in conjunction with a Kodak 23A Wratten red gelatin filter (Sigma Chemical Co., Poole, Dorset, UK) to provide the highest contrast. Using a filter frame holder (Sigma Chemical Co., Poole, Dorset, UK) the filters were orientated so that the red 23A filter was adjacent to the camera lens and the ultraviolet filter was between the light source and the red filter. In the photographs plasmids and chromosomes appeared as white bands against the dark background of the gel.

2.3.5 Estimation of plasmid size in the SPEAR isolates

Unknown plasmid sizes were estimated by comparing their mobilities with those of the reference plasmids which were included in each gel. Figure 7 shows a photograph of gel 69 (a typical medium-size agarose gel) run on the Horizon™ 11•14 electrophoresis equipment. Distances (mm) migrated by the plasmids from the edge of the well to the leading edge of the band were measured in the middle of the track on the Polaroid photographs viewed under an illuminated magnifier (Luxo, Gibco BRL, Paisley, Scotland). These distances were measured to the nearest 0·5 mm using a steel engineer's ruler (No. 147, England, UK). A plot of migration (distances in mm) against plasmid size (molecular weight in kb) for the reference plasmids gave a curved line. As interpolation is much more accurate if a function

can be found which gives a straight line [222], the natural logarithm of migration in mm was plotted against the natural logarithm of molecular weight in kb using the LINEST function [224 {p254}] in a spreadsheet designed for the purpose. The LINEST function uses the least squares method to calculate a straight line that best fits the data and returns an array that describes the line and provides regression statistics such as r^2 - the coefficient of determination. Calibration spreadsheets were calculated for each gel and the sizes (kb) of unknown plasmids were determined by applying the natural logarithms of their migration distances (mm) to the straight line equation on the spreadsheet. Figure 8 shows the spreadsheet for gel 69. The r^2 statistic, depicted in bold red italics in the LINEST array in Figure 8, compares estimated and actual y-values and ranges from 0 to 1. If it is 1, there is a perfect correlation in the sample i.e. there is no difference between the estimated y-value and the actual y-value. At the other extreme, if the r^2 statistic is 0, the regression equation is not helpful in predicting a y-value [224].

2.4 Transconjugation experiments: test and control systems

2.4.1 Introduction

The aim of the transconjugation experiments was to determine whether resistance of any of the SPEAR isolates to cefotaxime could be transferred to a recipient organism by conjugation. Each SPEAR isolate would be regarded as a potential donor organism in broth-mating experiments with a suitable recipient bacterium which was sensitive to cefotaxime.

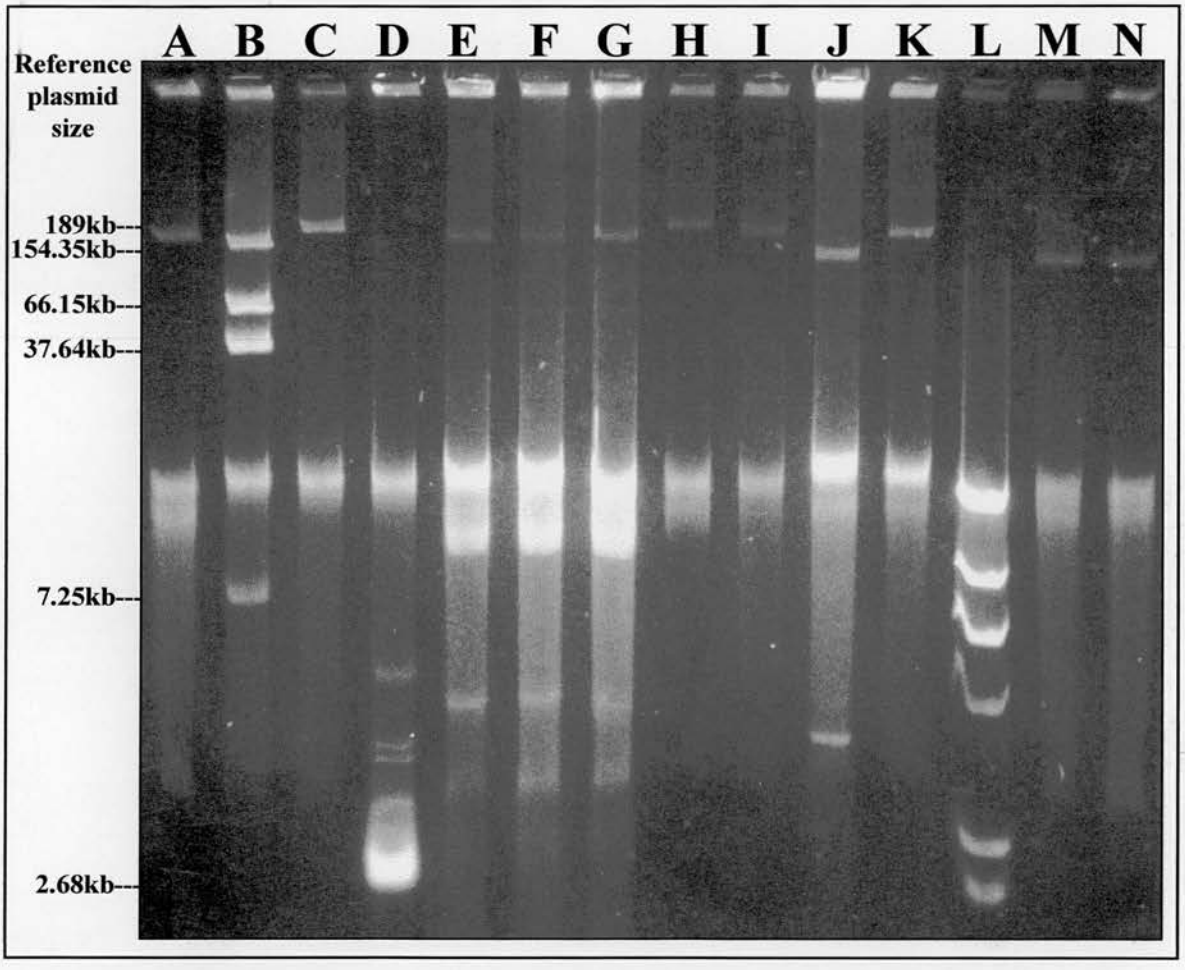

Figure 7 Agarose gel 069: Lane B - four reference plasmids from strain 39R861. Lane C - RTS 1 reference plasmid. Lane D - pUC9 reference plasmid (the sizes of the reference plasmids are shown on the left). Lane L - λDNA/*Hin*d III fragments. Lanes A, E to K, M and N - plasmid bands from SPEAR isolates 141, 100, 101, 102, 116, 121, 126, 134, 146 and 147.

Enter calibration values			Gel No. 069			
Size (kb)	Migration (mm)	Size (log kb)	Migration (log mm)	-0.382587698	4.396592196	2.391164271
189	11.0	5.24175	2.39790	0.007971507	0.030615325	2.468647563
154.35	12.0	5.03922	2.48491	***0.99826649***	0.030381545	2.792813301
66.15	16.5	4.19192	2.80336	2303.462292	4	3.008538273
37.64	19.5	3.62807	2.97041	2.126183911	0.003692153	3.638685404
7.25	37.0	1.98100	3.61092			4.019430818
2.68	57.5	0.98582	4.05178			
	Enter unknown plasmid values			Gel No. 069		
Isolate No.	Migration (mm)	Migration (log mm)	Calculated Size (log kb)	Calculated Size (kb)		
T	11.0	2.39790	5.22415	185.70		
T	12.0	2.48491	4.99673	147.93		
T	16.5	2.80336	4.16436	64.35		
T	19.5	2.97041	3.72771	41.58		
T	37.0	3.61092	2.05358	7.80		
T	57.5	4.05178	0.90125	2.46		
141	11.5	2.44235	5.10797	165.33		
100	12.0	2.48491	4.99673	147.93		
100	35.0	3.55535	2.19883	9.01		
100	46.0	3.82864	1.48450	4.41		
101	12.0	2.48491	4.99673	147.93		
101	35.0	3.55535	2.19883	9.01		
101	46.0	3.82864	1.48450	4.41		
102	12.0	2.48491	4.99673	147.93		
102	35.0	3.55535	2.19883	9.01		
102	46.0	3.82864	1.48450	4.41		
116	11.0	2.39790	5.22415	185.70		
121	11.5	2.44235	5.10797	165.33		
126	13.0	2.56495	4.78751	120.00		
126	47.0	3.85015	1.42829	4.17		
134	11.5	2.44235	5.10797	165.33		
146	13.0	2.56495	4.78751	120.00		
147	13.0	2.56495	4.78751	120.00		

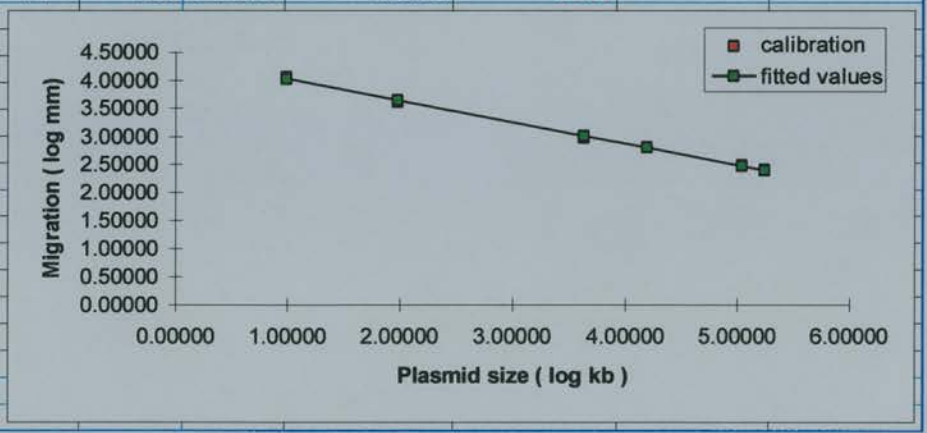

Figure 8 Calibration of agarose gel 069 with a log - log least squares straight line fit using Microsoft Excel® spreadsheet functions 'LINEST' and 'TREND'. The 'r^2 statistic' (in bold red italics above) gives an indication of how well the straight line equation explains the relationship between the variables. Unknown plasmid sizes are then read off using this equation.

If resistance to cefotaxime were transferable from a SPEAR donor isolate to the recipient bacterium then the cefotaxime-sensitive recipient bacterium would acquire resistance to cefotaxime, becoming a cefotaxime-resistant transconjugant. Such a transconjugant would be counterselected on a solid medium containing two antibiotics: cefotaxime which would inhibit the growth of the recipient organism and another antibiotic which would inhibit growth of the donor bacterium.

It would be necessary to confirm the identity of any putative transconjugant and to distinguish it from spurious overgrowth of either the donor or recipient organism on the selective plates. A transconjugant would be identical morphologically and biochemically (API 20 E system) to the recipient bacterium but would be resistant to cefotaxime. Finally the putative transconjugant would undergo agarose gel electrophoresis as previously described in order to determine whether it had acquired a plasmid from the donor SPEAR isolate to account for its resistance to cefotaxime.

In order to carry out the transconjugation experiments a suitable recipient bacterium had to be chosen which was sensitive to cefotaxime, with an MIC at least fourfold less than each of the SPEAR isolates, but resistant to another antibiotic to which all the SPEAR isolates were sensitive. For this purpose it was necessary to determine for each SPEAR isolate, i.e. each potential donor isolate, the MICs of any antibiotics which might be used to counterselect for transconjugants. The choice of recipient bacterium lay between two strains of *Escherichia coli*: either DH5α (kindly provided by Dr Russell Thompson, Institute of Virology, University of Glasgow) derived from DH5α ™ Competent Cells (Gibco BRL, Paisley, Scotland, UK) which was resistant to nalidixic acid or J62-2, a strain of *Escherichia coli* SA 128 (kindly provided by Professor SGB Amyes) which was resistant to rifampicin. Ideally the MIC of the chosen antibiotic (nalidixic acid or rifampicin) for the recipient bacterium would be at least four times greater than that for each donor isolate to minimize overgrowth on the selective plates.

Cefotaxime MICs for *Escherichia coli* DH5α and *Escherichia coli* J62-2 were <0.5 mg/L respectively by standard agar dilution antimicrobial susceptibility test [194] as already described.

2.4.2. Determination of rifampicin and nalidixic acid MICs for all SPEAR (potential donor) isolates and for the two potential recipient bacteria

A standard agar dilution antimicrobial susceptibility test was used [194] as described already for the cefotaxime MICs in section 2.2.3. Doubling dilutions of nalidixic acid (Adatabs, Difco, UK) ranging from 32 mg/L to 0.5 mg/L and rifampicin (Sigma Chemical Co., Poole, Dorset, UK) ranging from 125 mg/L to 2 mg/L were prepared separately in Mueller-Hinton agar (CM337, Oxoid, UK) at a temperature of approximately 50°C. Aliquots of each dilution were poured into sterile 85 mm plastic petri dishes (Sterilin, UK) on a level horizontal surface to a depth of 3-4 mm. Methanol was used as the solvent for the rifampicin powder [225] to make up the stock solution (25 mg/mL) prior to agar dilution. Standard inocula were prepared from the SPEAR isolates exactly as described before for the cefotaxime MICs and applied this time to the nalidixic acid and rifampicin plates. *Escherichia coli* NCTC 10418 was again used as the control organism and Mueller-Hinton agar without antibiotic was used as the growth medium control. Nalidixic acid and rifampicin MICs for each isolate were read as the lowest concentrations of each antibiotic causing inhibition of visible growth after overnight incubation at the optimal temperature (37°C or 30°C) for the organism [194].

Provided the MICs were appropriate the rifampicin-resistant *Escherichia coli* J62-2 was favoured as the recipient organism rather than the nalidixic acid-resistant strain for the following reasons. Nalidixic acid acts by inhibiting the action of DNA gyrase which is necessary for the supercoiling of chromosomal DNA in bacteria in order to have efficient cell division [226]. Resistant cells must be able to overcome this effect of nalidixic acid. Mutations in a number of chromosomal loci have been

described, resulting in DNA gyrases that are resistant to nalidixic acid. Many of these mutations involve the substitution of single amino acids at key enzymatic sites that are involved in the generation of the DNA gyrase-bacterial complex [226,227]. These mechanisms might exert an excessive strain on the cells, making them less likely to accept a plasmid. Also the DH5α nalidixic acid-resistant strain of *Escherichia coli* grew less well at 30°C, the optimal growth temperature for nine of the SPEAR isolates (isolates 13, 15, 18, 28-32 and 95). In fact the nalidixic acid MIC for *Escherichia* DH5α was only 32 mg/mL at 30°C while that for the nine SPEAR isolates which grew optimally at 30°C ranged from 16 to > 32 mg/L. Therefore *Escherichia coli* DH5α would not have been a suitable recipient for use at 30°C whereas the rifampicin MIC for *Escherichia coli* J62-2 was not affected by the lower temperature.

Using an extended range of cefotaxime dilutions from 1 mg/L to 0.015 mg/L the cefotaxime MIC for *Escherichia coli* J62-2 was found to be 0.125 mg/L by the method already described [194].

Using an extended range of rifampicin (Sigma Chemical Co., Poole, Dorset, UK) dilutions from 2000 mg/L to 16 mg/L the rifampicin MIC for *Escherichia coli* J62-2 was determined by the method described in section 2.2.3 to be 1000 mg/L [194].

The results of the rifampicin, nalidixic acid and cefotaxime MICs for the SPEAR isolates (potential donors) and the potential recipients, *Escherichia coli* J62-2 and *Escherichia coli* DH5α indicated that rifampicin would indeed be suitable as the counterselecting antibiotic and that the rifampicin-resistant *Escherichia coli* J62-2 would be satisfactory as the recipient bacterium in the transconjugation experiments with the SPEAR isolates (Appendix II). Further testing of the nalidixic acid-resistant *Escherichia coli* DH5α was not carried out.

Rifampicin was used at a concentration of 500 mg/L in the selective plates as some of the rifampicin MICs for the SPEAR isolates were 62 mg/L. Rifampicin BP with polysorbate and water solvent (Marion Merrell Dow Ltd, Lakeside House,

Stockley Park, Uxbridge, Middlesex, England) was available from the hospital pharmacy department in more appropriate amounts (600 mg vials) and much more cheaply than that obtainable from the Sigma Chemical Co. (150 mg vials). As standard parenteral preparations are not routinely recommended for susceptibility testing [194] direct comparison of MICs for *Escherichia coli* J62-2 and a laboratory reference strain *Escherichia coli* NCTC 10418 was made using each source of rifampicin in two standard agar dilution susceptibility tests [194] run in parallel. Rifampicin BP was reconstituted using the polysorbate solvent provided (Marion Merrell Dow Ltd) at a concentration of 100 mg/mL and rifampicin powder (Sigma Chemical Co.,Poole, Dorset, UK) dissolved in methanol (25 mg/mL) as described above. Dilutions ranged from 2000 mg/L to 2 mg/L for each source of rifampicin. As the results for each organism were identical using either source of rifampicin, Rifampicin BP (Marion Merrell Dow Ltd) was used in the preparation of the selective plates for the transconjugation experiments.

2.4.3 Preparation of selective plates for the test transconjugation system

Selective plates were prepared by adding suitably reconstituted Rifampicin BP (Marion Merrell Dow Ltd) and cefotaxime (Adatabs, Mast Laboratories, Bootle, Merseyside) to molten Mueller-Hinton agar at about 50°C to give respective concentrations of 500 mg/L and either 4 mg/L or 1 mg/L. Cefotaxime was used at a concentration of 4 mg/L in all cases other than six in which SPEAR isolates 6, 58, 76, 82, 138 and 143 were the potential donors. As their cefotaxime MICs were 8, 8, 4, 8, 8 and 8 mg/L respectively cefotaxime was used at a concentration of 1 mg/L for these isolates. The agar was then poured as quickly as possible to prevent cooling and partial solidification in the mixing container into 85 mm diameter Petri dishes on a level surface to a uniform depth of 3 to 4 mm. The agar containing the antibiotics was allowed to solidify at room temperature and the plates were either used immediately or stored at 4°C and used within 72 hours.

2.4.4 Broth mating in the test transconjugation system

One hundred and seventy-three of the 175 SPEAR isolates were examined in the test transconjugation system. A standard broth mating procedure was used (Professor SGB Amyes, personal communication). In the test system the potential donor was always one of the SPEAR isolates and the recipient was always *Escherichia coli* strain J62-2. The purity of each isolate was checked by plating out for single colonies on nutrient agar (CM271, Oxoid, UK) and incubating overnight at the appropriate temperature which was either 37°C, or 30°C when the donor was one of the twelve SPEAR isolates which grew optimally at the latter temperature.

Donor and recipient bacteria were grown separately overnight in 10 mL nutrient (Isosensitest) broth (CM473, Oxoid, UK)at 37°C or 30°C according to the optimal growth temperature of the SPEAR isolate. Then 0·1 mL of each donor broth was added to 4·5 mL of sterile nutrient broth pre-warmed to 37°C or 30°C and 1 mL of the recipient broth added to the same. Care was taken to avoid shaking the mating mixtures which were incubated overnight at 37°C or 30°C. Fifteen mL sterile phosphate buffered saline (PBS) pH 7.4 (Pharmacy Department, Western Infirmary) were added to the broth-mating mixtures, whirlimixed and centrifuged at 10,000 rpm for 15 min in 30 mL polypropylene tubes (Centritubes, Eastmoor, Runcorn, Cheshire) in an IEC Centra-3S bench centrifuge (Damon/IEC UK Ltd., Dunstable, Bedfordshire).

Each of the resulting bacterial pellets was resuspended in 0·5 mL PBS. Selective Mueller-Hinton agar plates containing rifampicin and cefotaxime as described above were allowed to equilibrate at ambient temperature then dried in an incubator at 37°C for 15 min with their lids ajar. A 0.1 mL amount of each resuspended pellet was spread over half of a selective plate then streaked out for single colonies using a standard loop. The plates were then incubated overnight at 37°C or 30°C as appropriate for the donor organism.

Donor and recipient bacteria were each plated out on selective plates (on which they were not expected to grow) and on Mueller-Hinton agar plates without antibiotics to act as growth controls. These plates were incubated overnight at 37°C and at 30°C. Following incubation any organism growing on the selective plates, i.e. any putative transconjugant, was investigated to check its identity. The test transconjugation system is depicted in Table 1(a).

2.4.5 The control broth transconjugation system

A control transconjugation system was devised to be run in parallel with every set of test transconjugation experiments. The aim of the control transconjugation system was to find out whether a control system donor, *Escherichia coli* CT 73, would transfer its plasmid coding for ampicillin resistance to the rifampicin-resistant recipient strain *Escherichia coli* J62-2, under the same general experimental conditions as those of the test system, the latter consisting of a SPEAR isolate acting as a potential donor with the same strain of *Escherichia coli* J62-2 as recipient. The control broth mating system was set up along with every set of test transconjugation experiments, including those carried out at 30°C, in order to check the viability of the procedure. The donor organism, *Escherichia coli* CT 73 (Figure 9), kindly donated by Dr Chris Thomson, (Bayer plc, Newbury, UK) had been isolated from a urinary tract infection. *Escherichia coli* CT 73 was able to transfer its plasmid-mediated resistance to ampicillin readily (Dr Chris Thomson, personal communication). The recipient strain *Escherichia coli* J62-2 (Figure 9) was the same organism as that used in the test system. Ideally ampicillin MICs would be at least fourfold less for the recipient than for the donor and rifampicin MICs would be at least fourfold greater for the recipient than for the donor bacterium.

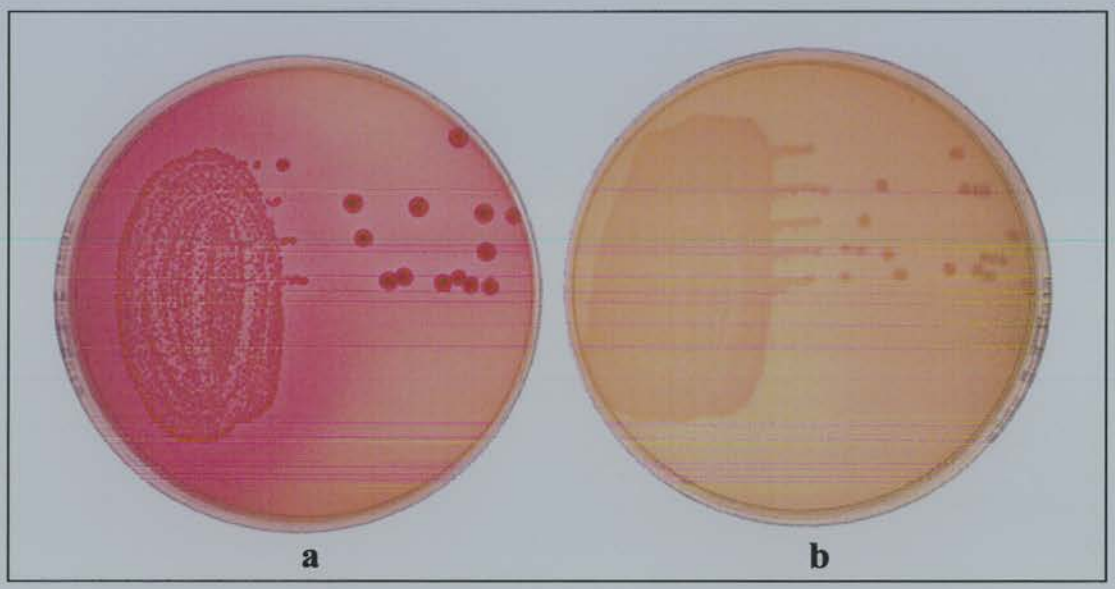

Figure 9 a) The donor lactose-fermenting *Escherichia coli* CT 73 and b) the recipient non-lactose-fermenting *Escherichia coli* J62-2 growing on MacConkey agar.

Ampicillin MICs were determined for *Escherichia coli* CT 73 and for *Escherichia coli* J62-2 by a standard agar dilution antimicrobial susceptibility method [194] using Adatabs (Mast Laboratories Limited, Bootle, Merseyside) to prepare dilutions of ampicillin ranging from 64 mg/L to 0.125 mg/L. The MIC of ampicillin for *Escherichia coli* CT 73 was >64 mg/L and that for *Escherichia coli* J62-2 was 4 mg/L. The MIC of rifampicin for *Escherichia coli* CT 73 was 8 mg/L and that for J62-2 was 1000 mg/L as determined by the method described in section 2.4.2. The results showed that a control transconjugation system with these strains would be possible using ampicillin and rifampicin for counterselection of transconjugants.

2.4.6 Preparation of selective plates for the control transconjugation system

It was decided to counterselect for transconjugants in the control system using ampicillin at a concentration of 16 mg/L which would inhibit the growth of the recipient, and rifampicin at a concentration of 500 mg/L which was greatly in excess of that needed to inhibit the growth of the donor. Rifampicin could have been used at a much lower concentration e.g. 32 mg/L in the control system, but in order to make the control conditions as similar as possible to the test conditions, it was used throughout at a concentration of 500 mg/L exactly as in the test system.

Plates for counterselection of transconjugants were prepared by adding suitably reconstituted Rifampicin BP (Marion Merrell Dow Ltd, Uxbridge, Middlesex) and ampicillin (Adatabs, Mast Laboratories, Bootle, Merseyside) to molten Mueller-Hinton agar held at 50°C in a water bath to give respective concentrations of 500 mg/L and 16 mg/L The agar was then poured as quickly as possible to prevent cooling and partial solidification in the mixing container into 85 mm diameter Petri dishes on a level surface to a uniform depth of 3 to 4 mm. The agar was allowed to solidify at room temperature and the plates were either used immediately or stored at 4°C and used within 72 hours. Each time the same batches of Rifampicin BP and Mueller-Hinton agar were used in the control and test systems.

(a) Test transconjugation system

		RP 500mg/L	CTX 4mg/L*
Potential donor	SPEAR isolate	S	R
Potential recipient	*Escherichia coli* J62-2	R	S
Potential transconjugant		R	R

RP - rifampicin, CTX - cefotaxime, S - sensitive, R - resistant.

(b) Control transconjugation system

		RP 500mg/L	AMP 16mg/L
Potential donor	*Escherichia coli* CT73	S	R
Potential recipient	*Escherichia coli* J62-2	R	S
Potential transconjugant		R	R

RP - rifampicin, AMP - ampicillin, S - sensitive, R - resistant.

(* in 167 SPEAR isolates, 1 mg/L was used in 6 SPEAR isolates with MICs of 4 or 8 mg/L. Two SPEAR isolates with MICs <0.5 mg/L were tested with ceftazidime in the additional test transconjugation system)

Table 1 Donor, recipient and transconjugants in (a) the test system and (b) the control system.

2.4.7 Broth mating in the control system

The methods were exactly as described above for the test system except that the donor was always *Escherichia coli* strain CT 73. The recipient was *Escherichia coli* strain J62-2 as in the test system. The control conjugation system was set up in parallel with every test transconjugation experiment. Transconjugants were colonies of *Escherichia coli* strain J62-2 rendered resistant to ampicillin by acquisition of a plasmid from *Escherichia coli* strain CT 73. The control transconjugation system is depicted in Table 1(b).

Putative transconjugants on the selective plates were replated on MacConkey agar (CM7, Oxoid, Basingstoke, UK) and incubated overnight at 37°C. Their sensitivity to ampicillin and other antibiotics was tested by a standard disk diffusion method as described previously [192] on Mueller-Hinton agar (CM337, Oxoid, UK) and compared with that of the recipient strain *Escherichia coli* J62-2 and the donor strain *Escherichia coli* CT 73. Antibiotic disks (Oxoid, UK), included: ampicillin 10 μg (AMP 10), oxacillin 1 μg (OX 1), cephalexin 30 μg (CL 30), erythromycin 15μg (E 15), trimethoprim 5 μg (W 5) and penicillin G 10 units (P 10). Putative transconjugants were identified by the API 20 E system and their biochemical profiles compared with those of the donor and recipient strains.

2.4.8 Plasmid detection in transconjugants from the control transconjugation system

Following antibiotic sensitivity testing and species identification as described above, transconjugants obtained in the control mating system were plated out on nutrient agar (CM271, Oxoid, UK) and incubated at 37°C along with purity plates of the donor strain *Escherichia coli* CT 73 and the recipient strain *Escherichia coli* J62-2. Preparations of plasmid DNA were made from the donor, the recipient and their transconjugants and agarose gel electrophoresis in the GNA-100 mini-gel apparatus carried out using methods described in detail in section 2.3.

2.5 ß-Lactamase analysis

2.5.1 Preparation of ß-lactamases from test isolates

All SPEAR isolates were tested for the production of ß-lactamases. Organisms were checked for purity by plating out for single colonies on nutrient agar (CM271, Oxoid, UK) and incubating overnight at 37°C or 30°C according to their optimal growth temperature. The tops of four or five colonies of each isolate were then touched with a sterile loop, inoculated on to nutrient agar slopes (CM271, Oxoid, UK) in bijoux and incubated overnight at 37°C or 30°C. Bacterial cells on each slope were removed with 1 mL of 10 mM sodium phosphate buffer [228] at pH 7·0, transferred to a sterile polypropylene microfuge tube (Treff microtubes, Scotlab, Bellshill, Scotland) and disrupted by sonication using an MSE Soniprep 150 Ultrasonic Disintegrator (© Sanyo Gallenkamp PLC, Meridian Business Park, Leicester, UK) fitted with an exponential microprobe. In order to minimise loss of ß-lactamase activity due to temperature increase during sonication each microtube, clamped in position, was immersed in a beaker of ice and water placed on the adjustable platform of the sonicator. Sonication was carried out using two 30-second pulses with a 30-second pause between pulses, after which the microtube was placed in a bucket of ice. Debris was removed by centrifugation (MSE Micro-Centaur Centrifuge, MSE, Loughborough, Leicestershire, UK) at 11,600 *g* for 15 minutes. The supernatant, which contained the ß-lactamase, was then transferred to a sterile microtube and subjected to isoelectric focusing or stored at minus 20°C.

2.5.2 Preparation of ß-lactamases used as pI control standards

Control ß-lactamases whose pIs were known were prepared from their carrier strains in exactly the same way as described for the test (SPEAR) isolates. These control standards included TEM-1 (pI 5·4); TEM-2 (pI 5·6); SHV-1 (pI 7·6); OXA-1 (pI 7·4); PSE-1 (pI 5·7); PSE-2 (pI 6·1) all kindly provided by Dr DM Livermore,

Central Public Health Laboratory, Colindale Avenue, London and OXA-4 (pI 7·5) kindly provided by Professor SGB Amyes, Department of Medical Microbiology, Medical School, University of Edinburgh. Carrier strains were *Escherichia coli* K12 J62-1 (TEM-1); *Escherichia coli* K12 J53-1 (SHV-1, OXA-1); *Pseudomonas aeruginosa* PU21 (TEM-2, PSE-1, PSE-2) and *Escherichia coli* K12 (OXA-4).

2.5.3 Spot-testing of ß-lactamase preparations with nitrocefin

In order to optimise the concentration of each ß-lactamase preparation applied to the gel spot-testing of each extract prior to loading on the gel was carried out using the chromogenic cephalosporin 87/312 [229] as described previously, marketed commercially as nitrocefin (Oxoid, UK). For this purpose nitrocefin was reconstituted according to the manufacturer's instructions, then diluted 1 in 10 using 10 mM phosphate buffer at pH 7·0.

Using a micropipette a 20 µL drop of diluted nitrocefin was placed in a sterile plastic Petri dish on a piece of white paper. Using a fresh sterile pipette tip a 20 µL drop of one of the ß-lactamase preparations was added to the drop of nitrocefin and mixed by drawing the material up and letting it down once or twice within the micropipette tip. The transparent Petri dish was convenient because many preparations could be tested in one dish and the colour change was easy to read against the background of white paper.

A colour change in the nitrocefin from pale yellow to pink within 20 to 60 seconds indicated that the ß-lactamase preparation could be used neat at a volume of 20 µL whereas a change of colour happening immediately or in less than 20 seconds suggested that the preparation should be diluted. Dilutions of 1 in 2, 1 in 3 or 1 in 4 were carried out easily by placing a 20 µL drop of the ß-lactamase extract in a sterile Petri dish and adding one, two or three 20 µL drops as required of 10 mM [228] sodium phosphate buffer [203 {pB.21}] at pH 7·0 using a fresh sterile pipette tip. A 20 µL drop of the appropriate dilution was then loaded on the gel (see below).

If the colour change did not develop within 60 seconds then a larger volume of that particular ß-lactamase preparation was loaded on to the gel using two or at most three sample application pieces stacked or laid end to end.

2.5.4 ß-Lactamase induction

If preparations did not react in spot-testing, the isolates from which they were derived were plated out on Mueller-Hinton agar (CM337, Oxoid, UK) and a 30 µg cefoxitin disk (Oxoid, UK) applied [230]. After appropriate incubation the colonies growing nearest to the disk were harvested and ß-lactamases again prepared as previously described.

2.5.5 Isoelectric focusing

2.5.5.1 Introduction

Isoelectric focusing [231] is a method of separation in which proteins align themselves as sharp bands at their pIs in an electrophoretically-produced pH gradient. A high degree of resolution is obtained by the method because isoelectric focusing is caused by forces that act against diffusion and proteins are therefore concentrated during their separation.

In 1975 Matthew and her colleagues [156] first described the use of analytical isoelectric focusing for detection and identification of ß-lactamases. Isoelectric focusing was carried out on sheets of polyacrylamide gel supported on glass plates and ß-lactamase was usually detected using a chromogenic cephalosporin as substrate. This compound, 3-(2,4-dinitrostyryl)-(6R,7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer, code number 87/312 [229] is one of a group of related cephalosporins which undergo a distinctive colour change when hydrolyzed by ß-lactamases. The solid is yellow and it gives a yellow solution when made up in water. It has an unusual absorption spectrum with an extra band of absorption with its λ_{max} at 386 nm. This is attributed to the high degree of

conjugation in the substituent in the 3-position and the further conjugation of this group with the ß-lactam ring and the double bond in the dihydrothiazine ring. This conjugation is thought to make the ß-lactam ring unusually reactive and the compound thus rapidly breaks down in the presence of ß-lactamases from a variety of bacteria.

O'Callaghan and colleagues [229] reported that the disruption of the ß-lactam ring was accompanied by a change in colour from yellow to red. While acknowledging that the rupture of the ß-lactam ring in cephalosporins was always accompanied by loss of the ultraviolet absorption in the 260 nm region they stated that a change in the visible region was unusual. Other cephalosporins conjugated in this way gave a similar effect, but if the 3-substituent did not carry a further chromophore, in this case two nitro groups, then the change in the spectrum was not visible and went undetected unless deliberately sought [229].

Following application of compound 87/312, focused bands with ß-lactamase activity appeared pink on a yellow background [156]. Enzymes that could not be distinguished biochemically or immunologically could be differentiated by analytical isoelectric focusing. Using this very sensitive technique the different enzymes produced by various strains each appeared as a pattern of bands (usually a group comprising a main band and satellites) which could be compared visually. Chromosomal and R factor-mediated ß-lactamases produced by a single strain appeared as separate entities [156].

2.5.5.2 Materials and Methods

ß-Lactamase preparations of all 175 SPEAR isolates were subjected to isoelectric focusing even if ß-lactamase had not been detected on spot-testing. In such cases the maximum amount of ß-lactamase preparation was loaded on to the gel.

Isoelectric focusing was carried out using a Multiphor II Electrophoresis System with a Multidrive™ XL power supply (both Pharmacia LKB Biotechnology,

Uppsala, Sweden) (Figure 10). The cooling plate was first positioned on the Multiphor II unit which was then bubble-levelled by adjustment of the levelling feet. Cold tap water was allowed to circulate through the cooling unit for 15 minutes before the run and throughout the procedure. Ampholine® PAGplates (Pharmacia LKB Biotechnology, Uppsala, Sweden), 1 mm thick polyacrylamide gels cast on a plastic support film, were used throughout the work in the pH range 3·5-9·5. In each case the gel was applied to the cooling plate with a uniform layer of non-charged insulating fluid to ensure efficient heat transfer from the gel during electrofocusing. To do this a few millilitres of 0.1% Triton X-100 non-ionic detergent solution were pipetted towards one end of the cooling plate. Starting at one end, the gel support was laid on the cooling plate over the detergent solution and gradually lowered to the horizontal position. When air bubbles became trapped, the gel was raised just enough to release the air then gradually lowered again on to the cooling plate. Any excess solution was removed with a tissue.

The electrode strips were wet uniformly with the appropriate electrode solution. The anode solution was 1 M H_3PO_4 stored in the dark at room temperature for up to six months and the cathode solution was 1 M NaOH which was stored at room temperature for up to one week only. To ensure that there were no dry spots caused by trapped air in the paper, the electrode strips were laid on a glass plate and using a pipette drawn along the strip, each one was wet with approximately 3 mL of the appropriate electrode solution. The strips were blotted to remove any excess electrode solution and each one laid approximately 2 mm from the edge of the gel at the cathode and anode, making sure that they were applied with the correct polarity. The ends of the strips were trimmed 2-3 mm short of the edge of the gel in order to avoid a short-circuit of the current path.

Figure 10 Power supply and electrophoresis equipment used in isoelectric focusing.

Samples were applied using sample application pieces (Pharmacia LKB Biotechnology) which were suitable for the application of 15-20 µL volumes. The dry pieces were applied with forceps to the Ampholine PAGplate surface approximately 10 mm from the cathode, although they could have been applied at any position in the pH gradient [156]. Using a micropipette and a fresh sterile pipette tip for each sample, 20 µL volumes of sample solution, previously spot-tested with nitrocefin as described above, were applied to each piece. When applying larger volumes of samples which had given a very weak reaction or had not reacted in the nitrocefin spot tests, two or at most three sample application pieces, stacked or laid end to end, were used.

At one end of the gel two 1 µL spots of lysed blood (100 mL defibrinated horse blood, E & O Laboratories, Bonnybridge, Scotland, lysed with 3 mL Saponin 20%, BDH Laboratories, Poole, England) were applied directly to the gel surface, opposite one another, approximately 10 mm from the cathode and anode respectively, to act as timing markers for the run [228].

The electrode holder was placed on the unit. Correct orientation of the electrodes with regard to polarity was checked. The electrodes were aligned with the centre of the electrode strips then the electrode holder was lowered carefully so that the electrodes rested on the electrode strips. After attaching the bridging socket to the pin connector the safety lid was placed in position and the electrode leads connected to the Multidrive™ XL power supply.

Running parameters set in the manual mode were 2000 V and 15 W constant power for approximately 1 hr 45 min. Usually the two bands derived from each of the spots of lysed blood met after approximately 80 min at which time the power supply was disconnected and the sample application pieces removed with forceps. The power supply was reconnected immediately and electrofocusing continued for a further 20 -30 minutes until the two bands from each of the spots of lysed blood had merged completely, then electrofocusing was stopped. The electrode strips were

lifted from the gel which was then removed from the cooling plate and nitrocefin applied as described below.

2.5.5.3 Detection of ß-lactamases and calculation of pI values

Nitrocefin (Oxoid, UK) made up as directed by the manufacturer was poured undiluted on to the gel and spread with a glass spreader. Within a few seconds pink bands began to appear. However as described by Matthew and colleagues [156] ß-lactamase bands visualised by this method appear gradually and in some cases by the time the weakest bands were visible the strongest bands had diffused. Therefore a water-resistant template (Pharmacia LKB Biotechnology) was aligned directly on the surface of the gel immediately after the application of nitrocefin and the position of the pink bands denoting the ß-lactamases marked with a pencil as soon as they appeared, as shown in Figure 11 for isoelectric focusing gel 9. After rinsing in tap water and drying between paper towels the template was photocopied for purposes of measurement and permanent record.

The distances in mm from the cathode to the pIs of the test and control ß-lactamases denoted by the pencil lines on each photocopied template were measured to the nearest mm using a steel engineer's ruler (No 147, England) and an illuminated magnifier (Luxo, Gibco BRL, Paisley, Scotland). The distances (mm) from the cathode to the pIs of the control ß-lactamases were then plotted against their known pI values using the Microsoft LINEST function [224] with unlogged data in a spreadsheet designed for the purpose. This function was described in detail in section 2.3.5 when it was used to determine plasmid sizes, except that logarithm/logarithm plots using the natural logarithms of the numerical values gave the best straight lines with the plasmid data.

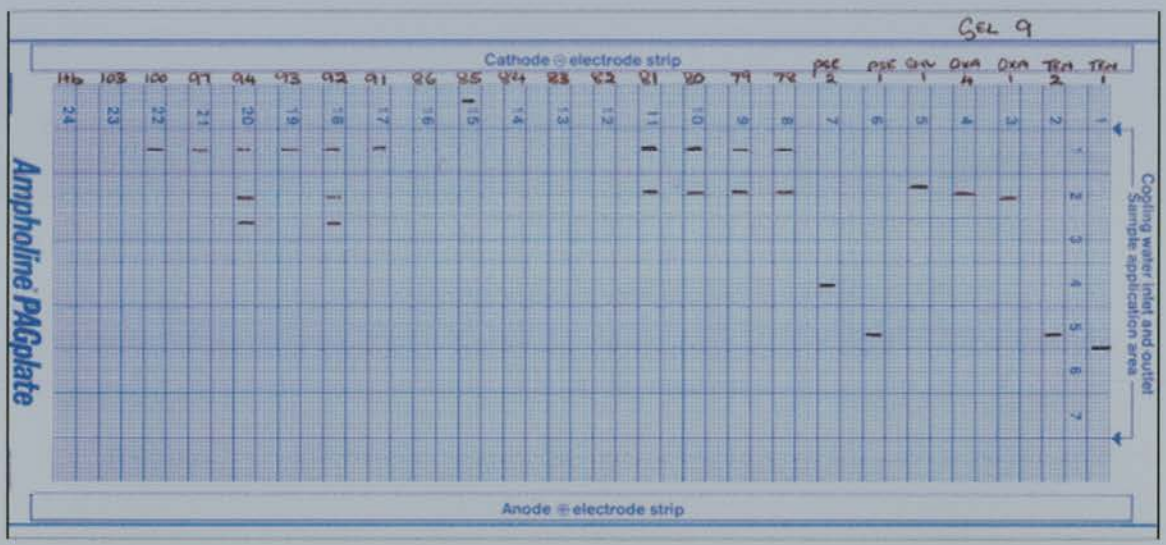

Figure 11 Water-resistant template placed on top of the isoelectric focusing gel to record the ß-lactamase bands directly as they developed (gel 9).

Calibration spreadsheets were calculated for each isoelectric focusing gel and the pI value of each unknown ß-lactamase was determined by applying the distance (mm) between the cathode and its pI to the straight line equation on the spreadsheet. Figure 12 shows the calibration spreadsheet for gel 9.

In order to keep a direct record of the raw data, photography of the gels was carried out using a Model QSP Quickshooter Camera fitted with a green filter and Model QSP No 27 Hood (Model QSP Quickshooter Photosystem, IBI Ltd, Clifton Road, Cambridge, UK). After application of nitrocefin each gel was transilluminated with fluorescent light on a portable Whatman Lightbox (Labsales, Maidstone, Kent, UK) and a photograph taken using Polaroid Type 665 black and white film ISO 80 (Sigma Chemical Co, Dorset, UK). Because of the fugitive nature of some of the bands it would have been necessary to take multiple serial photographs of each gel to obtain a complete photographic record as shown in Figure 13 for gel 9. Also for quantitative analysis it was more appropriate to record the ß-lactamase bands using the template method as described.

2.6 Additional transconjugation studies on four isolates which were positive in the Etest for ESBL production

Additional transconjugation studies were carried out on the two SPEAR isolates of *Klebsiella pneumoniae* (isolates 133 and 140) and two *Acinetobacter* isolates (132 and 137) which were positive in the Etest for ESBL production, using ceftazidime rather than cefotaxime along with rifampicin for counterselection.

2.6.1 Preparation of selective plates for the additional transconjugation studies

First the ceftazidime MIC for the recipient strain *Escherichia coli* J62-2 had to be determined by standard agar dilution antimicrobial susceptibility testing [194] as described already for the cefotaxime MICs.

pI Gel No.	9				
	Control pI	Migration from cathode edge (mm)	-16.34427032	150.4850637	62.22600392
TEM-1a	5.4	62	0.485840444	3.17521382	58.95714985
TEM-2a	5.6	59	***0.99560143***	1.173511936	29.53746327
OXA-1a	7.4	29	1131.732386	5	27.90303624
OXA-4a	7.5	28	1558.54292	6.885651322	26.26860921
SHV-1a	7.6	27			57.32272282
PSE-1a	5.7	59			50.78501469
PSE-2a	6.1	49			
Enter unknown beta-lactamase migration values to calculate pI					
Beta-lactamase No.	Migration from cathode edge (mm)	Calculated pI			
TEM-1a	62	5.4	pI Gel No.	9	
TEM-2a	59	5.6	pI Gel No.	9	
OXA-1a	29	7.4	pI Gel No.	9	
OXA-4a	28	7.5	pI Gel No.	9	
SHV-1a	27	7.6	pI Gel No.	9	
PSE-1a	59	5.6	pI Gel No.	9	
PSE-2a	49	6.2	pI Gel No.	9	
			pI Gel No.	9	
			pI Gel No.	9	
78	18	8.1	pI Gel No.	9	
78	27	7.6	pI Gel No.	9	
79	18	8.1	pI Gel No.	9	
79	27	7.6	pI Gel No.	9	
80	18	8.1	pI Gel No.	9	
80	27	7.6	pI Gel No.	9	
81	18	8.1	pI Gel No.	9	
81	27	7.6	pI Gel No.	9	
82	undetected	undetected	pI Gel No.	9	
83	undetected	undetected	pI Gel No.	9	
84	undetected	undetected	pI Gel No.	9	
85	7	8.8	pI Gel No.	9	
86	undetected	undetected	pI Gel No.	9	
91	18	8.1	pI Gel No.	9	
92	18	8.1	pI Gel No.	9	
92	27	7.6	pI Gel No.	9	
92	35	7.1	pI Gel No.	9	
93	18	8.1	pI Gel No.	9	
94	18	8.1	pI Gel No.	9	
94	27	7.6	pI Gel No.	9	
94	35	7.1	pI Gel No.	9	
97	18	8.1	pI Gel No.	9	
100	18	8.1	pI Gel No.	9	
103	undetected	undetected	pI Gel No.	9	

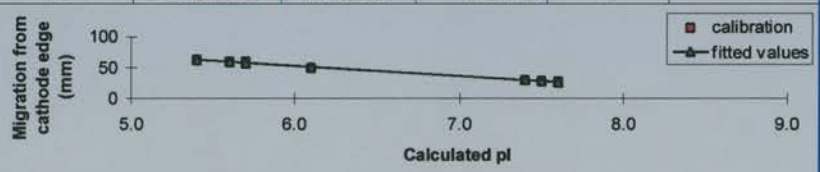

Figure 12 Calibration of isoelectric focusing gel 9 using a least squares straight line fit with Microsoft Excel® spreadsheet functions 'LINEST' and 'TREND'. The 'r^2 statistic' (in bold red italics above) gives an indication of how well the straight line equation explains the relationship between the variables. Unknown isoelectric points are read off this equation.

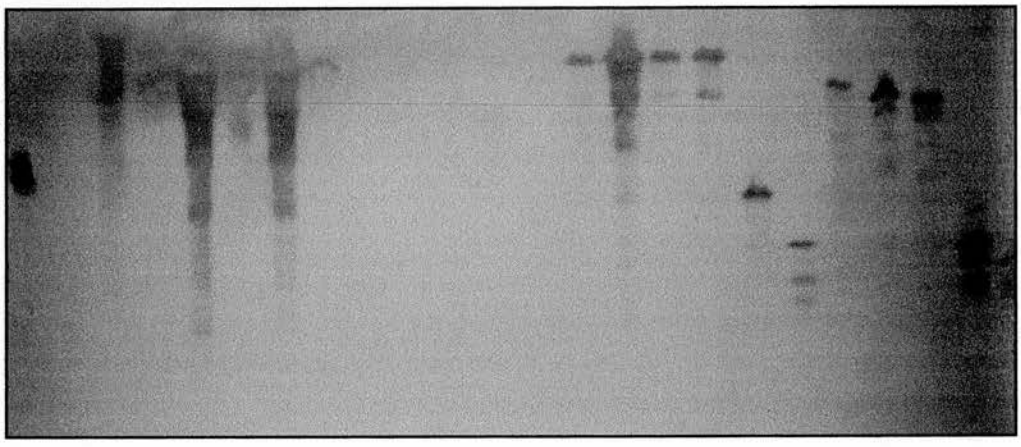

Figure 13 Photograph of isoelectric focusing gel 9

This time ceftazidime Adatabs (Mast Laboratories Ltd., Bootle, Merseyside, UK) were used to prepare doubling dilutions of ceftazidime ranging from 2 mg/L to 0.015 mg/L. The ceftazidime MIC for the recipient strain *Escherichia coli* J62-2 was 0.25 mg/L. Selective plates were prepared by adding reconstituted Rifampicin BP (Marion Merrell Dow Ltd) and ceftazidime (Adatabs, Mast Laboratories, Bootle, Merseyside) to molten Mueller-Hinton agar at about 50°C as described in section 2.4.3 to give rifampicin concentrations of 500 mg/L and ceftazidime concentrations of both 4 mg/L and 1 mg/L.

2.6.2 Broth mating in the additional transconjugation studies

The SPEAR potential donor isolates 133 and 140 (both *Klebsiella pneumoniae*), 132 (*Acinetobacter calcoaceticus* var. *anitratus*) and 137 (*Acinetobacter* species) were each grown in nutrient broth (CM271, Oxoid, UK), mated with recipient strain *Escherichia coli* J62-2 and the putative transconjugant material prepared and transferred to the selective plates containing rifampicin and ceftazidime. Donor and recipient bacteria were each plated out on selective plates (on which they were not expected to grow) and on Mueller-Hinton agar plates without antibiotics to act as growth controls. These plates were incubated overnight at 37°C. Following incubation any organism growing on the selective plates, i.e. any putative transconjugant, was investigated to check its identity.

2.6.3 Plasmid detection in transconjugants from the additional transconjugation studies

Plasmid preparations from transconjugants obtained in this additional test system were made and subjected to agarose gel electrophoresis as described in section 2.3.3 using the mini-gel GNA-100 (Pharmacia Molecular Biology, Uppsala, Sweden) and the Horizon™ 11•14 medium gel (Gibco BRL, Paisley, Scotland, UK) equipment. After staining with ethidium bromide as described in section 2.3.4 plasmids were visualised and photographed on a GDS 7500 Gel Documentation System

(Ultraviolet Products Ltd.) purchased from Genetic Research Instrumentation Ltd., Gene House, Rayne, Braintree, Essex, UK).

2.6.4 ß-Lactamase detection in transconjugants from the additional transconjugation studies

ß-Lactamases were prepared from transconjugants obtained in this additional test system. Isoelectric focusing and the calculation of pI values were carried out using the materials and methods already described in section 2.5.

Chapter 3 Results

3.1 Isolate collection

One hundred and seventy-five of the 200 SPEAR isolates collected were appropriate for inclusion in the study (Appendices I - V). They comprised *Acinetobacter calcoaceticus* var. *anitratus* (27), *Acinetobacter calcoaceticus* var. *lwoffii* (3), *Acinetobacter* species (1); *Citrobacter freundii* (10); *Enterobacter cloacae* (49), *Enterobacter* species (2); *Escherichia coli* (2); *Flavobacterium odoratum* (2); *Hafnia alvei* (4); *Klebsiella pneumoniae* (2); *Morganella morganii* (9); *Pseudomonas aeruginosa* (49), *Pseudomonas fluorescens*(1), *Pseudomonas species* (7) and *Stenotrophomonas maltophilia* (7). Of these 175 AGNB, 173 had cefotaxime MICs of 4 mg/L or greater. Both isolates of *Klebsiella pneumoniae* were sensitive to cefotaxime but resistant to ceftazidime and gentamicin. They were included in the study as possible ESBL producers.

3.2 Antimicrobial susceptibility testing

Results of agar dilution susceptibility tests (MICs) for cefotaxime and the disk diffusion and Etests are recorded in Appendices II III and IV. The results were interpreted (Table 2) according to NCCLS guidelines [192,194] apart from those for mecillinam (MEL 25) which, on the advice of the manufacturer (Leo Pharmaceuticals, Princes Risborough, Buckinghamshire, UK), were interpreted with reference to the work of Menday and Tybring [232]; those for temocillin (TEM 30) which were interpreted as recommended by SmithKline Beecham Pharmaceuticals, Welwyn Garden City, Hertfordshire, UK according to the work of Fuchs and colleagues [233] and those for meropenem (MEM 10) which were interpreted according to provisional guidelines issued by Zeneca Pharmaceuticals in 1994 [234 {p31}].

Antibiotic	(abbreviation)	Disk content	Zone Diameter (mm)			Equivalent MIC Breakpoints (mg/L)	
			R	I	S	R	S
Amoxycillin/clavulanic acid	(AMC 30)	20/10 µg	≤13	14-17	≥18	≥16/8	≤8/4
Ampicillin	(AMP 10)	10 µg	≤13	14-16	≥17	≥32	≤8
Azlocillin [a]	(AZL 75)	75 µg	≤17	---	≥18	≥128	≤64
Aztreonam	(ATM 30)	30 µg	≤15	16-21	≥22	≥32	≤8
Carbenicillin [a]	(CAR 100)	100 µg	≤13	14-16	≥17	≥512	≤128
Carbenicillin [b]	(CAR 100)	100 µg	≤19	20-22	≥23	≥64	≤16
Cefotaxime	(CTX 30)	30 µg	≤14	15-22	≥23	≥64	≤8
Cefoxitin	(FOX 30)	30 µg	≤14	15-17	≥18	≥32	≤8
Ceftazidime	(CAZ 30)	30 µg	≤14	15-17	≥18	≥32	≤8
Cephalexin	(CL 30)	30 µg	≤14	15-17	≥18	≥32	≤8
Ciprofloxacin	(CIP 5)	5 µg	≤15	16-20	≥21	≥4	≤1
Erythromycin	(E 15)	15 µg	not applicable			not applicable	
Gentamicin	(CN 10)	10 µg	≤12	13-14	≥15	≥8	≤4
Imipenem	(IPM 10)	10 µg	≤13	14-15	≥16	≥16	≤4
Mecillinam	(MEL 25)	25 µg	≤12	13-17	≥18	≥32	≤8
Meropenem	(MEM 10)	10 µg	≤11	12-13	≥14	≥16	≤4
Oxacillin	(OX 1)	1 µg	not applicable			not applicable	
Penicillin	(P 10)	10 units	not applicable			not applicable	
Piperacillin [a]	(PIP 100)	100 µg	≤17	---	≥18	≥128	≤64
Piperacillin [b]	(PIP 100)	100 µg	≤17	18-20	≥21	≥128	≤16
Piperacillin/tazobactam [a]	(TZP 110)	100/10 µg	≤17	---	≥18	≥128/4	≤64/4
Piperacillin/tazobactam [b]	(TZP 110)	100/10 µg	≤17	18-20	≥21	≥128/4	≤16/4
Temocillin	(TEM 30)	30 µg	≤15	16-18	≥19	≥32	≤16
Ticarcillin [a]	(TIC 75)	75 µg	≤14	---	≥15	≥128	≤64
Ticarcillin [b]	(TIC 75)	75 µg	≤14	15-19	≥20	≥128	≤16
Ticarcillin/clavulanic acid [a]	(TIM 85)	85 µg	≤14	---	≥15	≥128/2	≤64/2
Ticarcillin/clavulanic acid [b]	(TIM 85)	85 µg	≤14	15-19	≥20	≥128/2	≤16/2
Trimethoprim	(W 5)	5 µg	≤10	11-15	≥16	≥16	≤4

[a] when testing *Pseudomonas aeruginosa*

[b] when testing other Gram-negative organisms

R - Resistant

I - Intermediate

S - Susceptible

Table 2 Antibiotics, abbreviations and interpretative guidelines for disk susceptibility tests.

The results of the Etest for ESBL detection are also recorded in Appendix IV and were interpreted according to the manufacturer's instructions. When an ESBL-producing strain with a ceftazidime MIC equal to or greater than 1 mg/L is inhibited by clavulanic acid the MIC of ceftazidime is reduced by three or more two-fold dilutions when tested in the presence of clavulanic acid. A ratio of the MIC of ceftazidime 0.5 - 32 mg/L (ESBL_TZ) to the MIC of ceftazidime 0.064 - 4 mg/L with clavulanic acid 4 mg/L (ESBL_TZL) of less than 8 is a negative result whereas a ratio equal to or greater than 8 may indicate the presence of an inhibitable ESBL in certain Gram-negative bacteria (AB Biodisk, Solna, Sweden). When both MIC values are below or above the test ranges, interpretation is not determinable. Positive results were obtained for four isolates: 133 and 140 (*Klebsiella pneumoniae*) and 132 (*Acinetobacter calcoaceticus* var. *anitratus*) and 137 (*Acinetobacter* species). Ceftazidime MICs (ESBL_TZ) were recorded in Appendix IV as an integral part of the Etest for ESBL detection. Figures 14a and b show isolates 140 and 137 growing on nutrient agar and showing a positive result in the Etest for ESBL detection on Mueller-Hinton agar.

3.3 Plasmid analysis

Seventy-eight agarose gels were analysed as described in section 2.3.5 for gel 69 (Figures 7 and 8). Plasmids ranging from about (*c.*) 2 kb to *c.*214 kb were detected in 78 of the 175 SPEAR isolates. Forty-seven of the 78 isolates had multiple plasmids. The number '0.99826649' depicted in bold red italics in Figure 8 is the r^2 statistic, or coefficient of determination, as described in detail in section 2.3.5. The r^2 statistic ranged from 0.9915 to 0.9999 in 76 of the 78 gels. In only two of the gels was the value below 0.9915 (0.9868 and 0.9713). The percentage error for each reference plasmid was obtained by calculating the percentage of its actual size represented by the difference between its estimated and actual size.

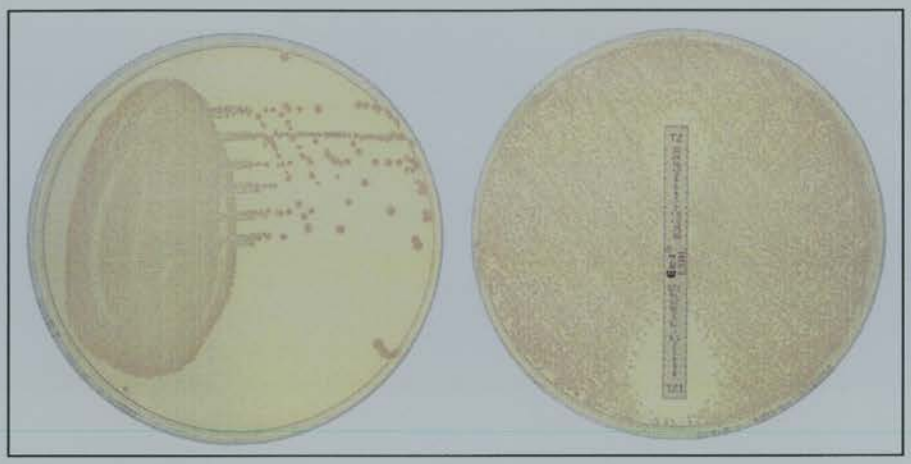

Figure 14 a Isolate 140 (*Klebsiella pneumoniae*) growing on nutrient agar (left) and showing a positive result in the E test for ESBL production on Mueller-Hinton agar (right).

Figure 14 b Isolate 137 (*Acinetobacter* species) growing on nutrient agar (left) and showing a positive result in the E test for ESBL production on Mueller-Hinton agar (right).

The mean percentage error for each reference plasmid, determined by the analysis of the data from all 78 gels, was as follows: RTS-1 (189 kb) - ± 5.0%; the four reference plasmids from strain 39R861: 154.35 kb - ± 4.6%, 66.15 kb - ± 4.8%, 37.64 kb - ± 15.7%; pUC9 (2.68 kb) - ± 5.0%. The results of the plasmid analysis of each SPEAR isolate are displayed on an isolate, plasmid and ß-lactamase matrix (Appendix V). The very large plasmid pMG5 (491 kb) as described in section 2.3.2.2 was not run routinely, but it was detected on both mini- and medium gels under the same conditions as the test isolates.

3.4 ß-Lactamase analysis

Twenty-nine isoelectric focusing gels were calibrated exactly as described previously for gel 9 (Figures 11 and 12). The number '0.99560143' depicted in bold red italics in Figure 12 is the r^2 statistic, or coefficient of determination, as described in detail in section 2.3.5. The r^2 statistic ranged from 0.9690 to 0.9999 and only six gel calibrations were below 0.9830. The error incurred for each control ß-lactamase was obtained by comparing its calculated pI value from the spreadsheet for each gel with its actual pI value. The mean error for each control ß-lactamase determined by analysis of the data from all 29 gels was as follows: TEM-1 (pI 5.4) - ± 1.90%; TEM-2 (pI 5.6) - ± 0.50%; SHV-1 (pI 7.6) - ± 0.04%; OXA-1 (pI 7.4) - ± 0.07%; OXA-4 (pI 7.5) - ± 0.19%; PSE-1 (pI 5.7) - ± 0.11%; PSE-2 (pI 6.1) - ± 3.01%. ß-Lactamases of pI values ranging from 4.9 to 8.8 were detected in 147 of the 175 isolates and multiple enzyme bands were detected in 38 of these isolates. The results for each isolate are shown in Appendix V in an isolate, plasmid and ß-lactamase matrix.

3.5 Transconjugation experiments

3.5.1 Transconjugation in the test mating system

Mating experiments were set up between 173 of the 175 SPEAR isolates and *Escherichia coli* J62-2 using rifampicin and cefotaxime as the counterselecting antibiotics. Transferable resistance to cefotaxime was not detected in the test mating system.

3.5.2 Transconjugation in the control mating system

The control mating system was set up in parallel with every batch of SPEAR isolates in the test system. Transconjugants were obtained on the selective plates in the control system on each occasion, at 37°C and at 30°C. Figure 15 shows transconjugants growing on a Mueller-Hinton selective plate containing rifampicin 500 mg/L and ampicillin 16 mg/L. Transconjugants, like the recipient *Escherichia coli* J62-2, were non-lactose-fermenters on MacConkey agar and gave an identical biochemical profile to that of the recipient *Escherichia coli* J62-2 in the API 20 E system. Figures 16a, b and c show respectively the recipient, the donor and their transconjugants growing on MacConkey agar and on Mueller-Hinton agar with a selection of antibiotics. The recipient *Escherichia coli* J62-2 (non-lactose-fermenter) shows sensitivity to ampicillin, cephalexin and trimethoprim and of course resistance to oxacillin, erythromycin and penicillin (Figure 16a). The donor *Escherichia coli* CT 73 (lactose-fermenter) shows resistance to ampicillin, cephalexin, trimethoprim, oxacillin, erythromycin and penicillin (Figure 16b). The transconjugants (non-lactose-fermenters) show resistance to ampicillin, oxacillin, erythromycin and penicillin but sensitivity to cephalexin and trimethoprim (Figure 16c). The zone sizes (mm) obtained in these disk diffusion tests are shown in Table 3. Transconjugants comprised the recipient *Escherichia coli* J62-2 made resistant to ampicillin by acquisition of a plasmid from the donor *Escherichia coli* CT 73.

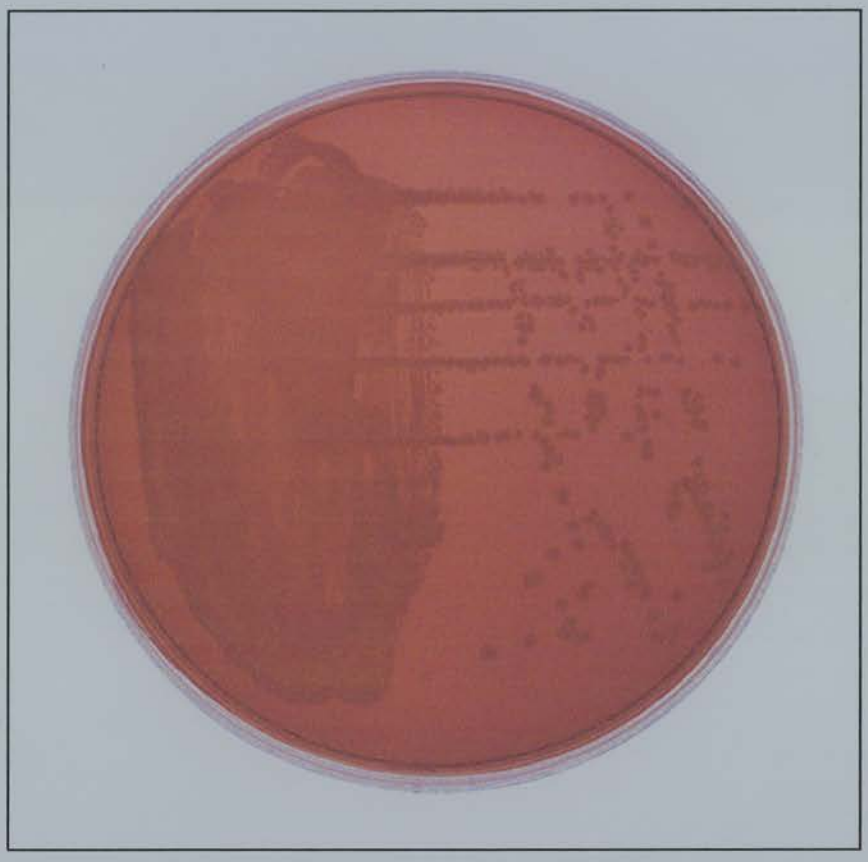

Figure 15 Transconjugants from the control mating system growing on Mueller-Hinton agar containing rifampicin 500 mg/L and ampicillin 16 mg/L.

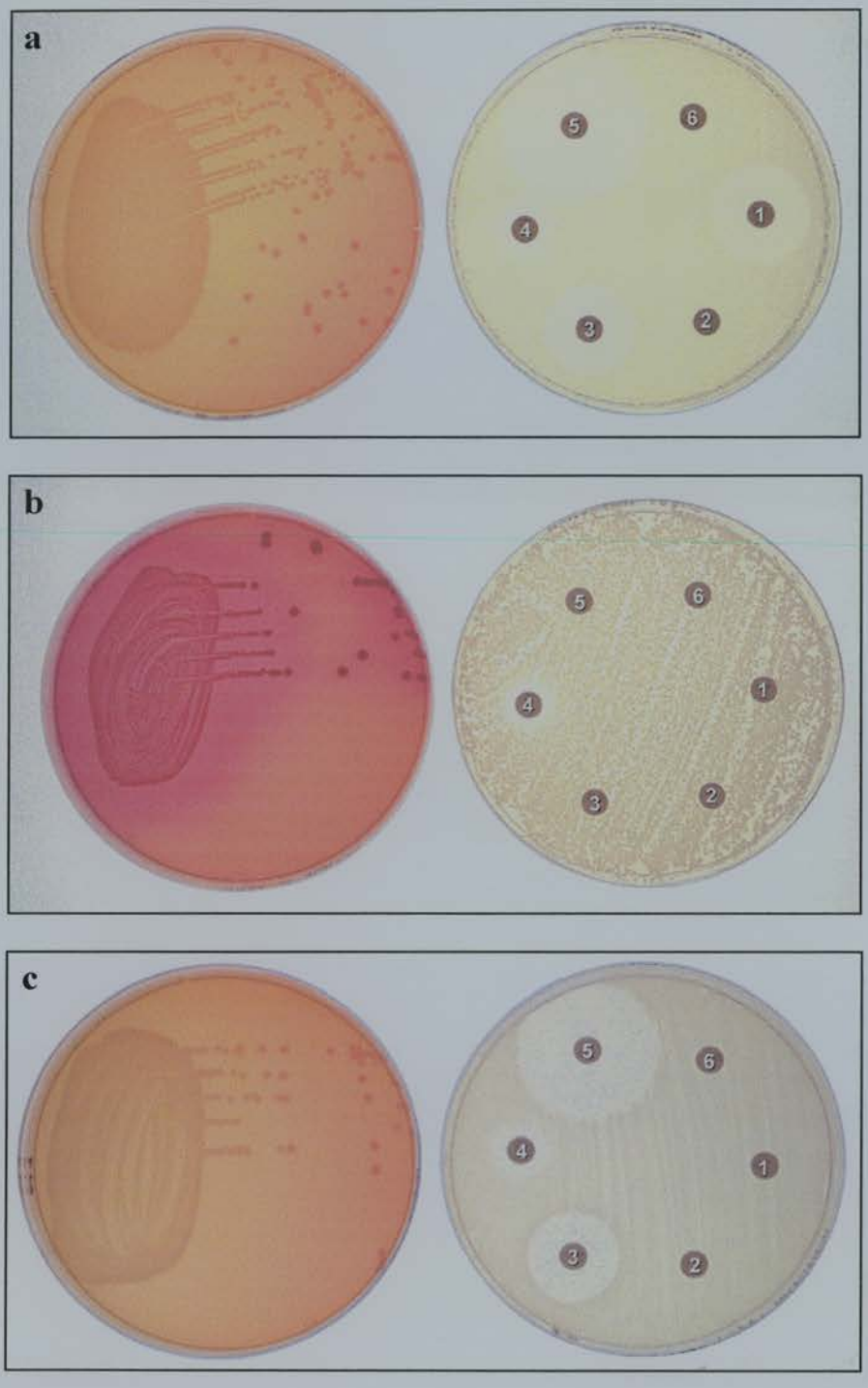

Figure 16 a) The recipient *Escherichia coli* J62-2, b) the donor *Escherichia coli* CT 73 and c) their transconjugants growing on MacConkey agar (left) and showing their individual sensitivity patterns on Mueller-Hinton agar (right). Antibiotic discs: 1-ampicillin, 2-oxacillin, 3-cephalexin, 4-erythromycin, 5-trimethoprim, 6-penicillin.

3.5.3 Plasmid detection in transconjugants from the control transconjugation system

Agarose gel electrophoresis of plasmid DNA prepared from the donor *Escherichia coli* CT 73, the recipient *Escherichia coli* J62-2 and their transconjugants is shown in Figure 17 for gel 127 and its calibration spreadsheet is shown in Figure 18. The donor *Escherichia coli* CT 73 had three plasmids of *c.*62 kb, 6 kb and 2 kb. Plasmids were not detected in the recipient *Escherichia coli* J62-2. Each transconjugant preparation had a plasmid band at the level of the largest donor plasmid (*c.*62 kb). The transfer of this plasmid was associated with ampicillin resistance in the transconjugants as described above.

3.6 Additional transconjugation studies on the two *Klebsiella* isolates and two *Acinetobacter* isolates which were positive in the Etest for ESBL detection

3.6.1 Transconjugants

Transconjugants were obtained from the *Klebsiella pneumoniae* isolates 133 and 140 using rifampicin 500 mg/L and ceftazidime 1 mg/L as the counterselecting antibiotics. Transconjugants were not detected when ceftazidime was used at the higher concentration of 4 mg/L. Transconjugants were not obtained from the *Acinetobacter* isolates at either ceftazidime concentration.

A useful practical feature in the transconjugation experiments was the distinctive "flat" morphology of the recipient *Escherichia coli* J62-2. (Figure 19). The purity of the putative transconjugants was rigorously checked, then antibiotic susceptibility testing and identification by the API 20 E system (bioMérieux, Marcy l'Etoile, France) were set up in each case. The biochemical profile of the transconjugants matched exactly that of the recipient strain *Escherichia coli* J62-2 and differed from that of the donor isolates (Figure 20).

Antibiotic / Isolate	AMP10	OX1	CL30	E15	W5	P10
Escherichia coli CT73	0	0	0	9	0	0
Escherichia coli J62-2	19	0	20	0	30	0
Transconjugant	0	0	20	0	30	0

(* antibiotic abbreviation code as in Table 2)

Table 3 Antibiograms for the donor, recipient and their transconjugants in the control transconjugation system.

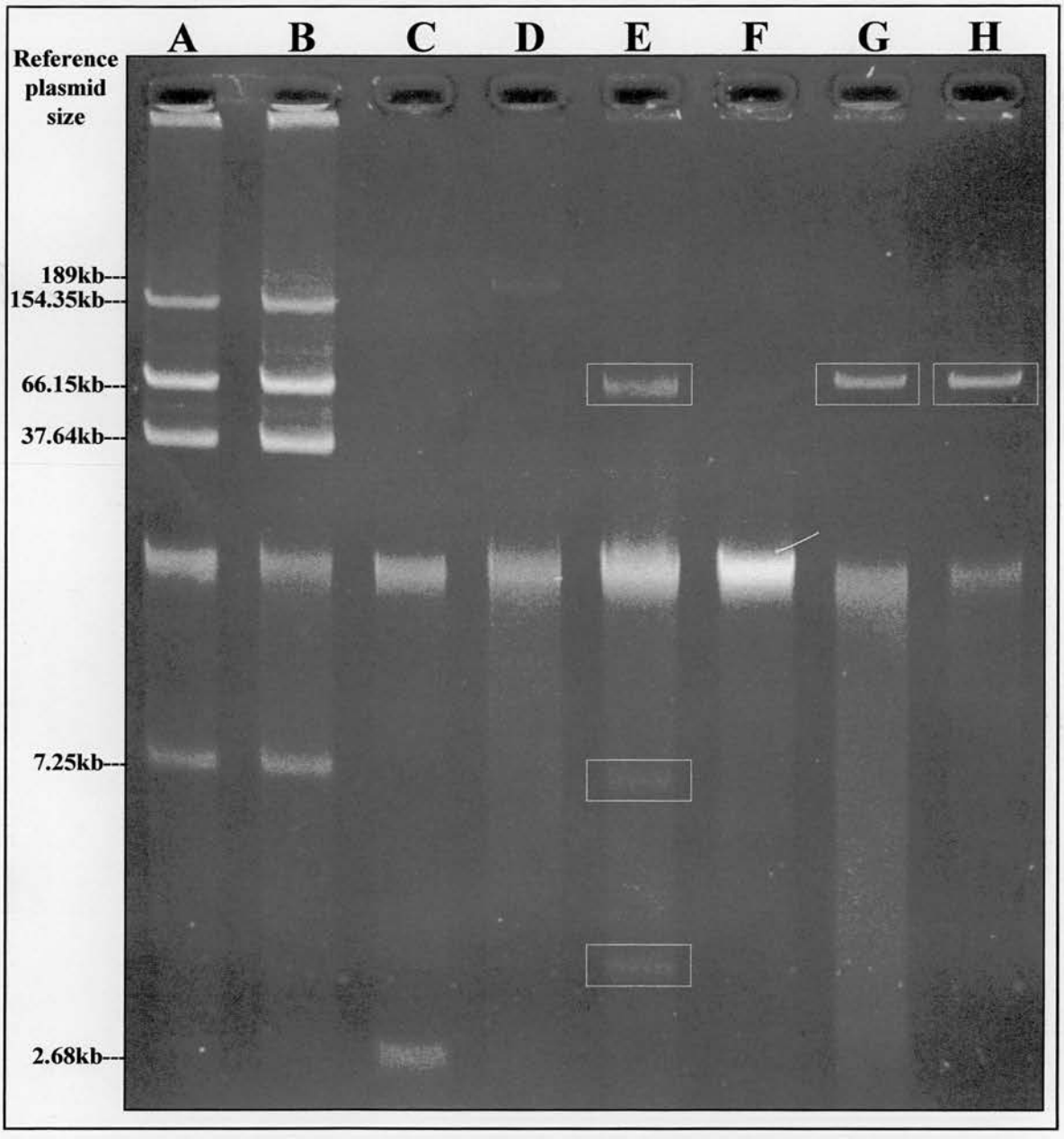

Figure 17 Agarose gel 127: Lanes A and B - reference plasmids from strain 39R861. Lane C - pUC9 reference plasmid. Lane D - RTS 1 reference plasmid (the sizes of the reference plasmids are shown on the left). Lane E - donor *Escherichia coli* CT 73 showng three plasmid bands (highlighted □). Lane F - recipient *Escherichia coli* J62-2 (no plasmids). Lanes G and H - transconjugants each showing one plasmid band (highlighted □) at the level of the largest plasmid (*c.*62 kb) of the donor representing transfer of this plasmid from the donor *Escherichia coli* CT 73.

Enter calibration values			Gel No. 127			
Size (kb)	Migration (mm)	Size (log kb)	Migration (log mm)	-0.353174496	4.347420706	2.496169346
189	12.0	5.24175	2.48491	0.007954153	0.030548674	2.567695751
154.35	13.0	5.03922	2.56495	***0.997975168***	0.030315403	2.866939746
66.15	18.0	4.19192	2.89037	1971.472835	4	3.066079861
37.64	21.0	3.62807	3.04452	1.811830194	0.003676095	3.647781511
7.25	40.0	1.98100	3.68888			3.999255357
2.68	53.0	0.98582	3.97029			

	Enter unknown plasmid values			Gel No. 127		
Isolate No.	Migration (mm)	Migration (log mm)	Calculated Size (log kb)	Calculated Size (kb)		
T	12.0	2.48491	5.27364	195.12		
T	13.0	2.56495	5.04700	155.55		
T	18.0	2.89037	4.12558	61.90		
T	21.0	3.04452	3.68911	40.01		
T	40.0	3.68888	1.86463	6.45		
T	53.0	3.97029	1.06783	2.91		
CT73	18.0	2.89037	4.12558	61.90		
CT73	41.0	3.71357	1.79472	6.02		
CT73	52.0	3.95124	1.12176	3.07		
J62-2	0.0	no plasmid	no plasmid	no plasmid		
Trans 1	18.0	2.89037	4.12558	61.90		
Trans 2	18.0	2.89037	4.12558	61.90		

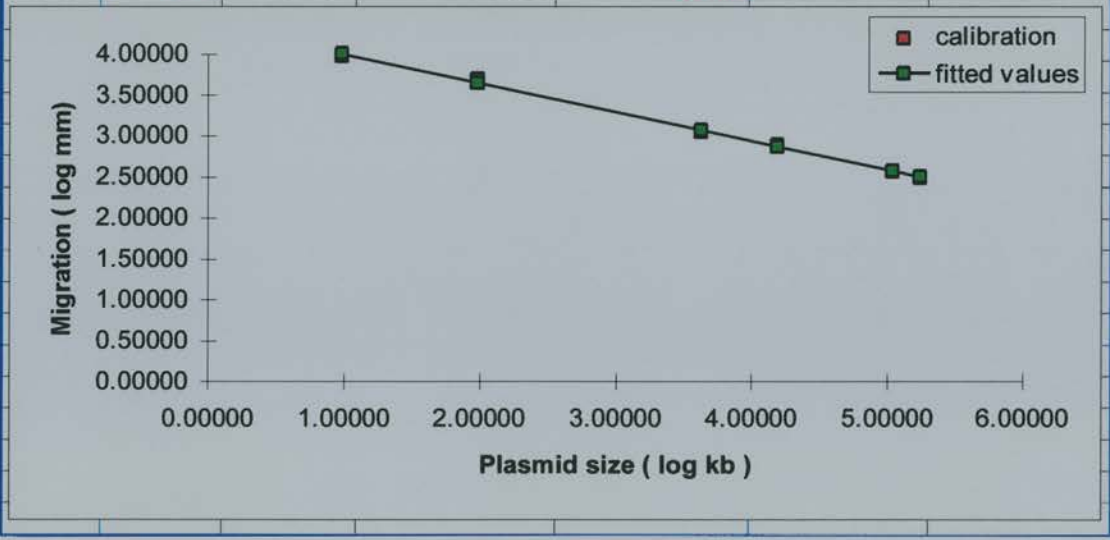

Figure 18 Calibration of agarose gel 127 with a log - log least squares straight line fit using Microsoft Excel® spreadsheet functions 'LINEST' and 'TREND'. The 'r^2 statistic' (in bold red italics above) gives an indication of how well the straight line equation explains the relationship between the variables. The size of the transferred plasmid was then read off using this equation and compared with the donor's plasmids.

Antibiograms for the donor *Klebsiella pneumoniae* isolate 140, the recipient *Escherichia coli* J62-2 and their transconjugants are shown in Table 4. These results are similar to those obtained for donor *Klebsiella pneumoniae* isolate 133 and its transconjugants (not shown). Transconjugants from both donor isolates 133 and 140 were themselves positive in the Etest for ESBL detection. Ratios of ESBL_TZ (MIC of ceftazidime alone) to ESBL_TZL (MIC of ceftazidime in the presence of clavulanic acid) were 3/0.125 = 24 for transconjugants of isolate 133 and 6/0.25 = 24 for transconjugants of isolate 140. "Phantom" inhibition zones indicative of ESBL production in the Etest were also recorded for transconjugants obtained from isolates 133 and 140.

3.6.2 Plasmid analysis of the *Klebsiellae* transconjugants

Plasmid preparations of the donor *Klebsiella pneumoniae* isolates 140 and 133, the recipient organism *Escherichia coli* J62-2 and their transconjugants were examined along with reference plasmid preparations in the GNA-100 (Pharmacia Molecular Biology, Uppsala, Sweden) mini-gel system as shown in Figure 21 (agarose gel 139). Figure 22 shows its calibrated spreadsheet. Bands representing large plasmids (*c.*160 kb) can be seen in preparations from both donor isolates 140 and 133. In the transconjugant preparation from isolate 140, only a band representing a plasmid size of *c.*214 kb can be seen. Another band representing a smaller plasmid (*c.*38 kb) can be seen nearer to the chromosomal band in each of the donor isolates 140 and 133. Plasmid bands are not seen in the transconjugant preparation from isolate 133. Similar results which are not shown were obtained in repeat examinations using six different plasmid preparations of the same isolates. The band representing the largest plasmid of isolate 140 varied in intensity between preparations and was "fuzzy" and ill-defined.

Organism	Zone diameters (mm) for each antibiotic*								
	CTX30	CN10	PRL100	AZL75	AMC30	FOX30	CIP5	IPM10	CAZ30
Donor: isolate 140 *Klebsiella pneumoniae*	36	0	12	0	11	28	34	30	16
Recipient: *Escherichia coli* J62-2	36	22	32	25	18	27	38	30	27
Transconjugants	34	0	14	0	17	27	40	34	19

Organism	Zone diameters (mm) for each antibiotic*								
	CAR100	TIC75	TIM85	AMP10	TZP110	ATM30	MEM10	MEL25	TEM30
Donor: isolate 140 *Klebsiella pneumoniae*	0	0	0	0	27	30	30	20	17
Recipient: *Escherichia coli* J62-2	25	25	24	18	28	32	33	32	20
Transconjugants	0	0	14	0	30	34	30	21	0

(* antibiotic abbreviation code as in Table 2)

Table 4 Antibiograms for the donor, recipient and their transconjugants in the additional transconjugation studies.

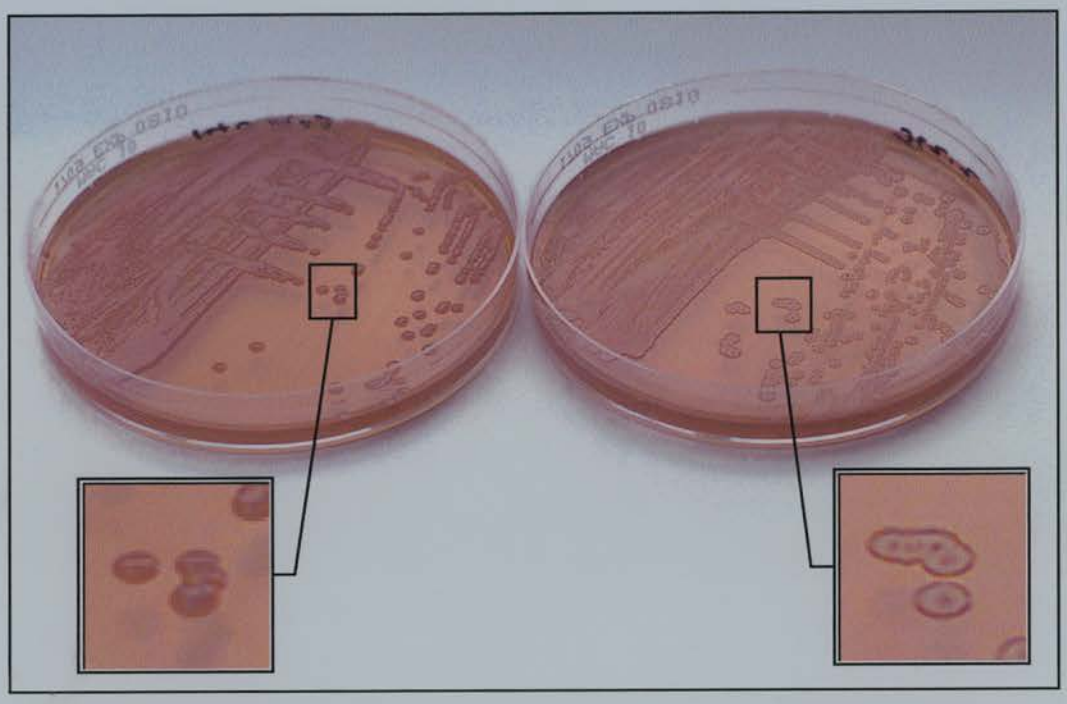

Figure 19 Donor isolate 140 (*Klebsiella pneumoniae*) and recipient *Escherichia coli* J62-2 growing on MacConkey agar. The distinctive morphology of the *Escherichia coli* J62-2 is shown on the right.

Figure 20 API 20 E biochemical profiles:
a) recipient *Escherichia coli* J62-2
b) donor *Klebsiella pneumoniae* (SPEAR isolate 140)
c) transconjugant (profile identical to that of the recipient).

Various plasmid preparations of donor isolate 140 and its transconjugants were examined in the Horizon™ 11•14 medium gel system (Gibco BRL, Paisley, Scotland, UK) as shown in Figure 23 (agarose gel 140) and Figure 24 shows its calibrated spreadsheet. Instead of one band representing a large plasmid in isolate 140, two bands can be seen representing plasmid sizes of *c.*214 kb and 160 kb respectively. The band representing a much smaller plasmid of *c.*38 kb can be seen nearer to the chromosomal band as before. A band representing a plasmid of *c.*214 kb can be seen in two of the transconjugant preparations from isolate 140 and very faintly (highlighted in Figure 23) in a third preparation which was more dilute. In another transconjugant preparation a band representing a plasmid of *c.*160 kb can be seen (highlighted in Figure 23). As the antibiograms of all the transconjugants of isolate 140 were identical, it may be that the plasmid band of *c.*214 kb represented the OC form of the 160 kb plasmid rather than another larger plasmid.

3.6.3 ß-lactamase analysis of the *Klebsiella* transconjugants

The results of isoelectric focusing of the donor *Klebsiella pneumoniae* isolates 133 and 140, the recipient strain *Escherichia coli* J62-2, their transconjugants and controls are shown in Figures 25 (isoelectric focusing gel 33) and 26 (calibration spreadsheet). Both donor *Klebsiella pneumoniae* isolates had a TEM-type enzyme which aligned with the control TEM-1 enzyme and an SHV-type enzyme which aligned with the control SHV-1 and SHV-2 enzymes. The recipient isolate *Escherichia coli* J62-2 had a ß-lactamase of pI 8.4 which was presumably chromosomally-mediated. The transconjugants of both isolates 133 and 140 each had the TEM-type enzyme of pI 5.2 and also the ß-lactamase of pI 8.4 which aligned with that of the recipient *Escherichia coli* J62-2 strain. They did not have the SHV-type enzyme. The band of pI 4.8 is probably an accessory band of the TEM-type enzyme.

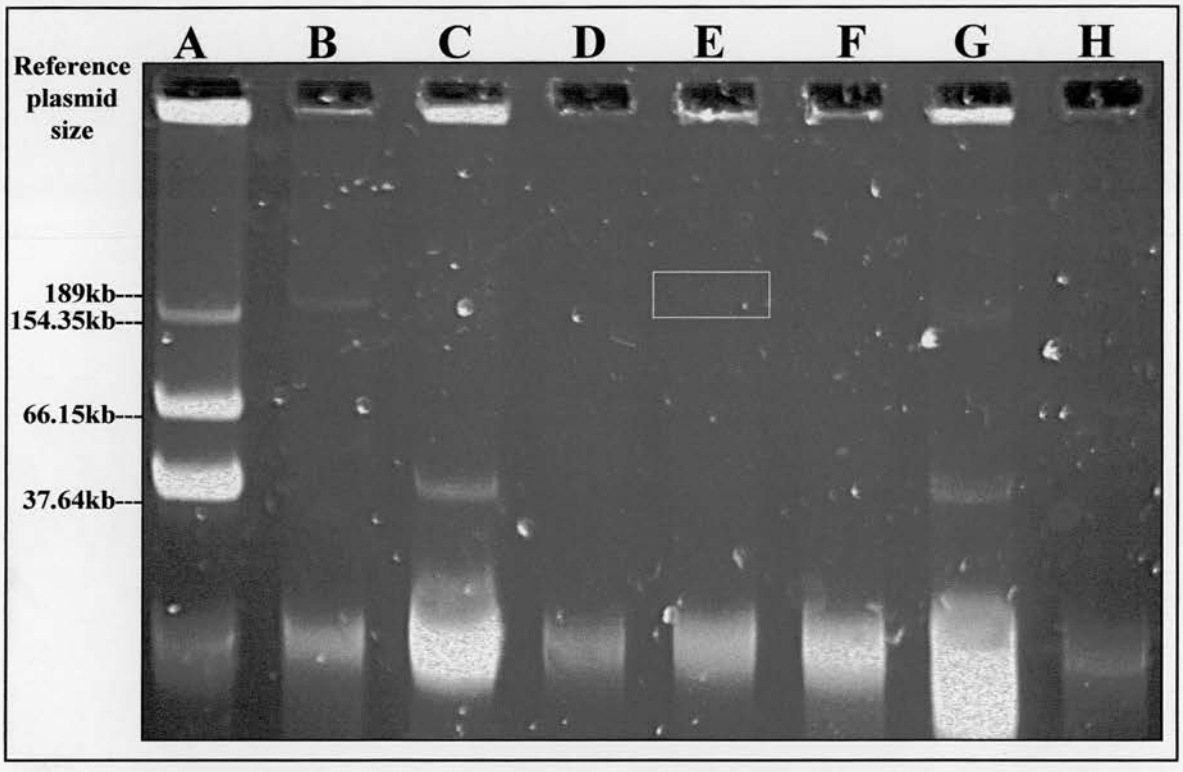

Figure 21 Agarose gel 139: Lane A - reference plasmids from strain 39R861. Lane B - RTS 1 reference plasmid. (the sizes of the reference plasmids are shown on the left). Lane C - donor *Klebsiella pneumoniae* isolate 140 with two plasmid bands at *c.*160 kb and 38 kb. Lane D - recipient *Escherichia coli* J62-2 with no plasmids. Lane E - transconjugants of donor isolate 140 with plasmid band at *c.*214kb (highlighted □). Lane F - dilute preparation of transconjugants of donor isolate 140 with no plasmid bands. Lane G - donor *Klebsiella pneumoniae* isolate 133 with two plasmid bands at *c.*160 kb and 38 kb. Lane H - transconjugant of donor isolate 133 with no plasmid bands.

Enter calibration values			Gel No. 139			
Size (kb)	Migration (mm)	Size (log kb)	Migration (log mm)	-0.384491828	4.89670678	2.881297889
189	18.0	5.24175	2.89037	0.011465696	0.052418668	2.959166813
154.35	19.0	5.03922	2.94444	***0.998224646***	0.014921176	3.284945916
66.15	27.0	4.19192	3.29584	1124.536123	2	3.501744546
37.64	33.0	3.62807	3.49651	0.250368405	0.000445283	#N/A
						#N/A

	Enter unknown plasmid values			Gel No. 139	
Isolate No.	Migration (mm)	Migration (log mm)	Calculated Size (log kb)	Calculated Size (kb)	
T	18.0	2.89037	5.21815	184.59	
T	19.0	2.94444	5.07753	160.38	
T	27.0	3.29584	4.16360	64.30	
T	33.0	3.49651	3.64169	38.16	
140	19.0	2.94444	5.07753	160.38	
140	33.0	3.49651	3.64169	38.16	
J62-2	0.0	no plasmid	no plasmid	no plasmid	
140TC	17.0	2.83321	5.36681	214.18	
133	19.0	2.94444	5.07753	160.38	
133	33.0	3.49651	3.64169	38.16	
133TC	0.0	no plasmid	no plasmid	no plasmid	
140TC2	0.0	no plasmid	no plasmid	no plasmid	

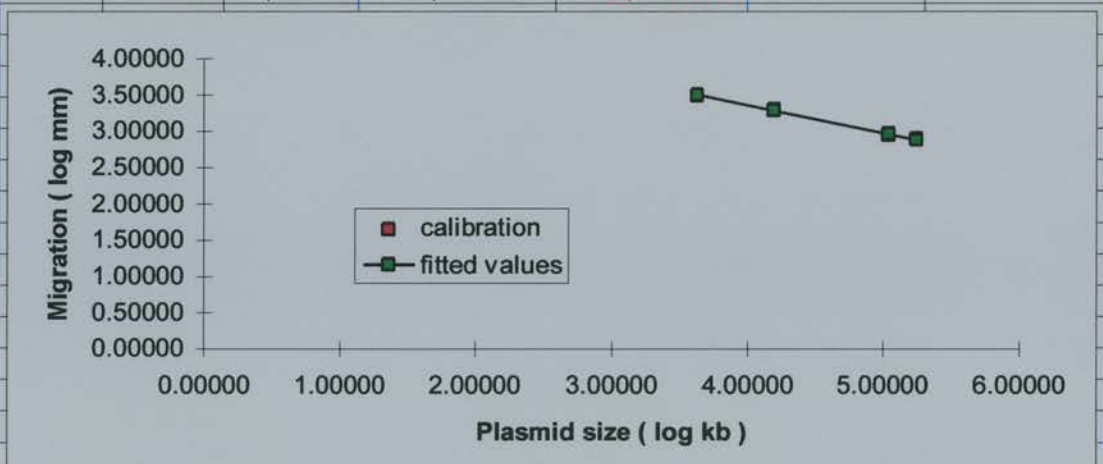

Figure 22 Calibration of agarose gel 139 with a log - log least squares straight line fit using Microsoft Excel® spreadsheet functions 'LINEST' and 'TREND'. The 'r^2 statistic' (in bold red italics above) gives an indication of how well the straight line equation explains the relationship between the variables. The sizes of the plasmids of donor isolates 133 and 140 and of the transconjugants of isolate 140 were then read off using this equation.

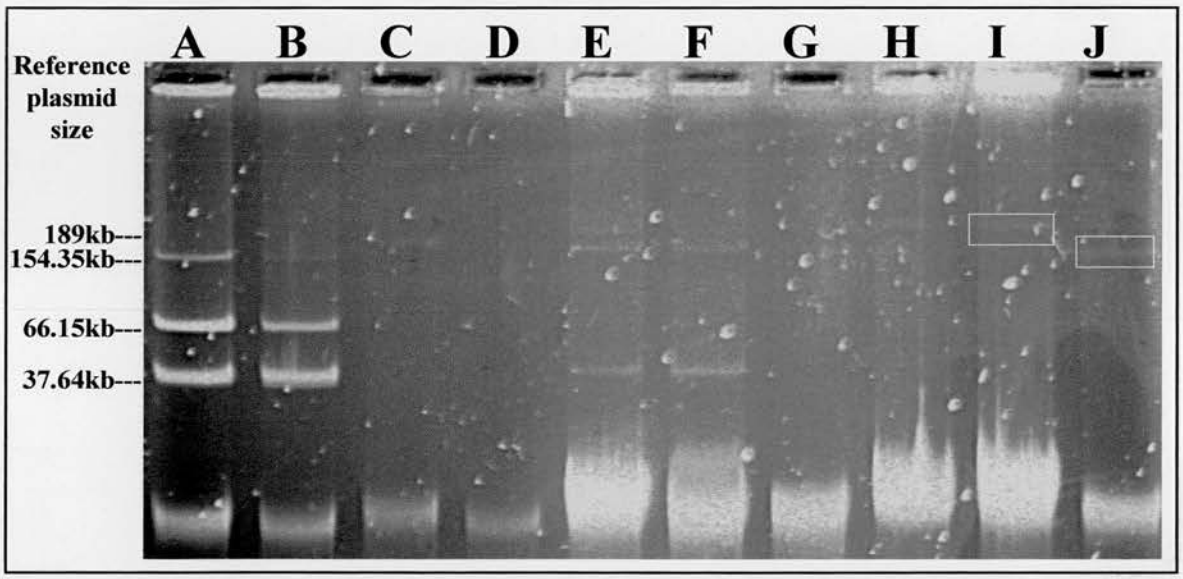

Figure 23 Agarose gel 140 showing plasmid transfer from the donor *Klebsiella pneumoniae* isolate 140 in the additional test transconjugation system: Lanes A and B - reference plasmids from strain 39R861. Lane C - RTS 1 reference plasmid (the sizes of the reference plasmids are shown on the left). Lane D - recipient *Escherichia coli* J62-2 with no plasmids. Lanes E and F - donor *Klebsiella pneumoniae* isolate 140 with three plasmid bands *c.* 214 kb, 160 kb, 38 kb. Lane G - 140 TC1* with no plasmid bands. Lane H - 140 TC2* with one plasmid band *c.* 214 kb. Lane I - 140 TC3* with one plasmid band *c.* 214 kb (highlighted □). Lane J - 140 TC4* with one plasmid band *c.* 160 kb (highlighted □).

* TC1, TC2, TC3, TC4 - different plasmid preparations of transconjugants of donor isolate 140.

Enter calibration values			Gel No. 140			
Size (kb)	Migration (mm)	Size (log kb)	Migration (log mm)	-0.366020464	4.558957072	2.640370399
189	14.0	5.24175	2.63906	0.013157498	0.060153223	2.714498424
154.35	15.0	5.03922	2.70805	***0.997422225***	0.017122847	3.02462678
66.15	21.0	4.19192	3.04452	773.8629894	2	3.231010191
37.64	25.0	3.62807	3.21888	0.226890353	0.000586384	#N/A
						#N/A
	Enter unknown plasmid values			**Gel No. 140**		
Isolate No.	Migration (mm)	Migration (log mm)	Calculated Size (log kb)	Calculated Size (kb)		
T	14.0	2.63906	5.24533	189.68		
T	15.0	2.70805	5.05684	157.09		
T	21.0	3.04452	4.13757	62.65		
T	25.0	3.21888	3.66122	38.91		
J62-2	0.0	no plasmid	no plasmid	no plasmid		
140	14.0	2.63906	5.24533	189.68		
140	15.5	2.74084	4.96726	143.63		
140	26.0	3.25810	3.55407	34.96		
140TC1	14.0	2.63906	5.24533	189.68		
140TC2	14.0	2.63906	5.24533	189.68		
140TC3	14.0	2.63906	5.24533	189.68		
140TC4	15.5	2.74084	4.96726	143.63		

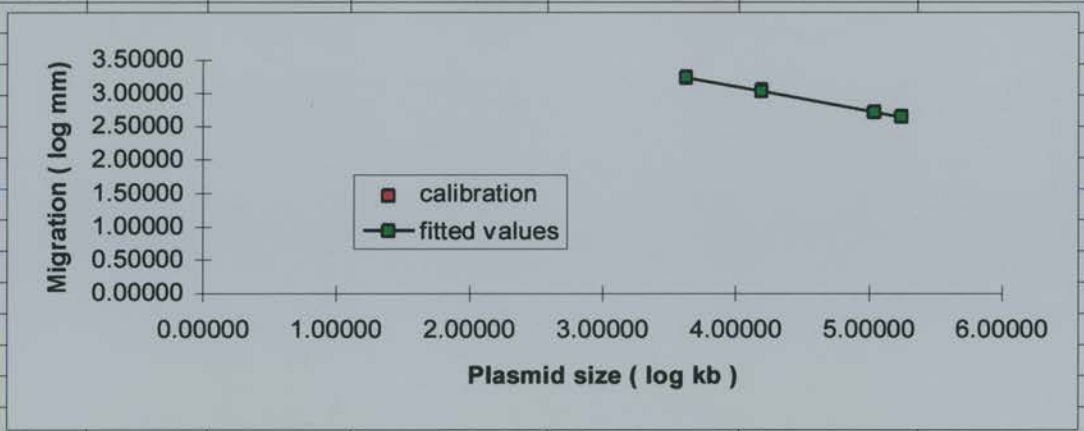

Figure 24 Calibration of agarose gel 140 with a log - log least squares straight line fit using Microsoft Excel® spreadsheet functions 'LINEST' and 'TREND'. The 'r^2 statistic' (in bold red italics above) gives an indication of how well the straight line equation explains the relationship between the variables. The sizes of the plasmids of donor isolate 140 and its transconjugants were then read off using this equation.

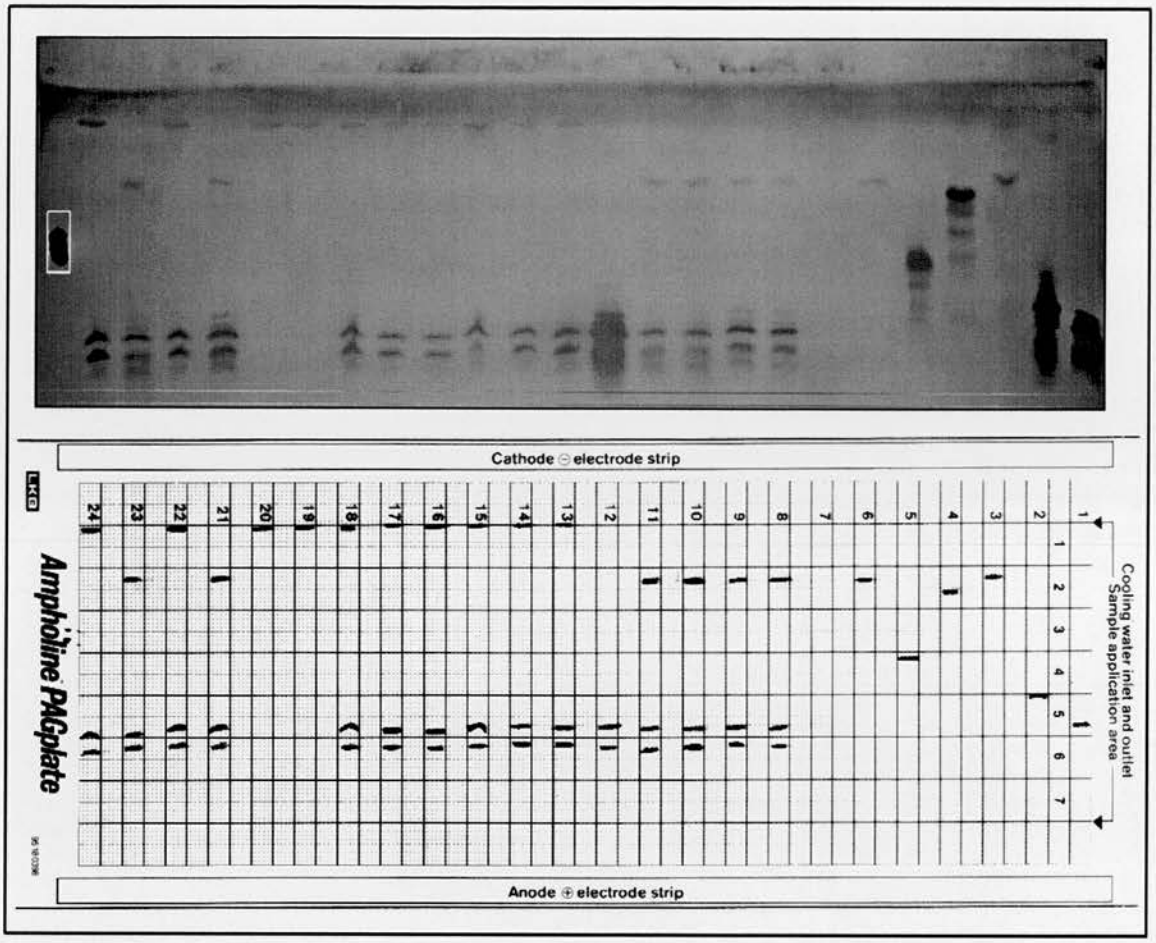

Figure 25 Isoelectric focusing gel 33 and its template showing the ß-lactamase bands from donors 133 and 140 (both *Klebsiella pneumoniae*), the recipient *Escherichia coli* J62-2 and their transconjugants (TC).

Lanes from right to left as shown on the template (1 - 24)

1 Tem-1 (control)	5 PSE-2 (control)	9 140
2 Tem-2 (control)	6 SHV-2 (control)	10 133
3 SHV-1 (control)	7 Blank	11 140
4 OXA-1 (control)	8 140TC	12 Tem-1 (control)
13 140TC	17 140TC	21 140
14 140TC	18 133TC	22 140TC
15 133TC	19 J62-2	23 133
16 140TC	20 J62-2	24 133TC

(Haemoglobin marker bands seen at extreme left of the gel - highlighted □)

pI Gel No.	33				
	Control pI	Migration from cathode edge (mm)	-14.50735064	135.9903034	57.65060995
TEM-1a	5.4	60	0.768326162	5.135059681	54.74913982
TEM-2a	5.6	54	***0.988904942***	1.773541333	25.73443854
SHV-1a	7.6	26	356.5208831	4	28.63590866
OXA-1	7.4	29	1121.418205	12.58179543	47.4954645
PSE-2	6.1	45			25.73443854
SHV-2	7.6	26			
Enter unknown beta-lactamase migration values to calculate pI					
Beta-lactamase No.	Migration from cathode edge (mm)	Calculated pI			
TEM-1a	60	5.2	pI Gel No.	33	0.988904942
TEM-2a	54	5.7	pI Gel No.	33	0.988904942
SHV-1a	26	7.6	pI Gel No.	33	0.988904942
OXA-1	29	7.4	pI Gel No.	33	0.988904942
PSE-2	45	6.3	pI Gel No.	33	0.988904942
SHV-2	26	7.6	pI Gel No.	33	0.988904942
140	26	7.6	pI Gel No.	33	0.988904942
140	61	5.2	pI Gel No.	33	0.988904942
140	66	4.8	pI Gel No.	33	0.988904942
133	26	7.6	pI Gel No.	33	0.988904942
133	61	5.2	pI Gel No.	33	0.988904942
133	66	4.8	pI Gel No.	33	0.988904942
TEM-1	61	5.2	pI Gel No.	33	0.988904942
TEM-1	66	4.8	pI Gel No.	33	0.988904942
140TC1	14	8.4	pI Gel No.	33	0.988904942
140TC1	61	5.2	pI Gel No.	33	0.988904942
140TC1	66	4.8	pI Gel No.	33	0.988904942
133TC	14	8.4	pI Gel No.	33	0.988904942
133TC	61	5.2	pI Gel No.	33	0.988904942
133TC	66	4.8	pI Gel No.	33	0.988904942
J62-2	14	8.4	pI Gel No.	33	0.988904942

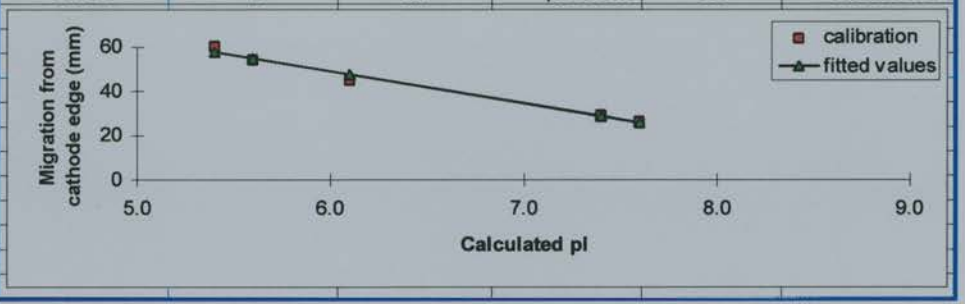

Figure 26 The pIs of the ß-lactamases of the donor *Klebsiella pneumoniae* isolates 133 and 140, the recipient *Escherichia coli* J62-2 and their transconjugants were read off a least squares straight line fit using Microsoft Excel® spreadsheet functions 'LINEST' and 'TREND'. The 'r^2 statistic' (in bold red italics above) gives an indication of how well the straight line equation explains the relationship between the variables.

3.7 Individual genera

3.7.1 *Acinetobacter*

Thirty-one isolates belonging to the genus *Acinetobacter* were examined. Twenty-seven were identified as *Acinetobacter calcoaceticus* var. *anitratus*, three as *Acinetobacter calcoaceticus* var. *lwoffii* (isolates 18, 46 and 123) and one (isolate 137) was identified satisfactorily to genus only. (More recently the genus *Acinetobacter* has been reclassified into 17 genomic species of which *Acinetobacter baumannii* is the one most commonly isolated from clinical material). Sixteen of the 31 isolates were obtained from patients on their first day of admission to ITU. Twenty of the 31 isolates contained plasmids ranging from *c.*3 kb to 142 kb. Most of these (22 of 35 plasmids) were under 30 kb. Thirteen isolates each had one plasmid, six had two plasmids and one had nine plasmids. ß-Lactamases with pI values between 5.4 and 8.8 were detected in 26 of 31 isolates.

All 31 isolates were susceptible to the carbapenems (imipenem and meropenem). Only one showed reduced sensitivity to ciprofloxacin and three were resistant to gentamicin. All but three showed reduced sensitivity or were resistant to mecillinam as expected and all but one were resistant to temocillin. Twenty-five of 31 isolates were sensitive to carbenicillin, ticarcillin and piperacillin. Twenty-nine of 31 isolates showed reduced sensitivity or were resistant to aztreonam. Only two of the 31 isolates were resistant to ceftazidime by disk diffusion testing but in the Etest for ESBL detection nineteen strains showed reduced sensitivity or were resistant to ceftazidime (MICs equal to or greater than 8 mg/L). Twenty-seven of the 31 isolates were resistant to cefoxitin by disk diffusion testing. Two isolates (132 and 137) were positive in the Etest for ESBL detection. All isolates were resistant to ampicillin. Six (3, 13, 18, 46, 88, 130) were resistant to amoxycillin/clavulanic acid and one (114) showed reduced sensitivity.

Isolates 3, 15, 18, 46 and 86 were resistant to carbenicillin and isolate 108 showed reduced sensitivity. Isolates 3, 18 and 46 were resistant also to ticarcillin and strains 15 and 86 showed reduced sensitivity. Isolates 3 and 130 were resistant to piperacillin and isolates 15, 46, 86. and 114 showed reduced sensitivity. However isolate 114 gave good inhibition zones to carbenicillin and ticarcillin and isolate 130 was sensitive to carbenicillin. Although isolate 18 was resistant to amoxycillin/clavulanic acid and ticarcillin it gave a good zone of inhibition with ticarcillin/clavulanic acid.

The two isolates most resistant to the ß-lactams were isolate 3 (*Acinetobacter calcoaceticus* var. *anitratus*) and isolate 46 (*Acinetobacter calcoaceticus* var. *lwoffii*) both of which showed reduced sensitivity to piperacillin/tazobactam and were resistant to most of the other ß-lactams: ampicillin, amoxycillin/clavulanic acid, carbenicillin, ticarcillin, ticarcillin/clavulanic acid, aztreonam, cefoxitin and ceftazidime. Isolate 46 showed reduced sensitivity to piperacillin and isolate 3 was resistant. Isolate 3 was sensitive to temocillin and isolate 46 was resistant. Both showed good zones of inhibition to mecillinam (unexpected for *Acinetobacter* species which are usually resistant to mecillinam) and to the carbapenems imipenem and meropenem and both were sensitive to ciprofloxacin and gentamicin. Isolate 3 had a small plasmid of *c.*9 kb and a ß-lactamase of pI 8.3. Isolate 46 had two plasmids of *c.*8 kb and 97 kb and a ß-lactamase of pI 8.3. The two isolates were not temporally or epidemiologically related.

Isolate 3 had been isolated from the tracheal aspirate of a 34-year-old patient (patient 20) on her twelfth day in ITU with recurrent Guillain-Barré syndrome. Ten days prior to her ITU admission she had begun treatment with benzylpenicillin for pneumococcal pneumonia. SPEAR was started on admission to ITU, cefotaxime being stopped after four days. Isolates of *Acinetobacter calcoaceticus* var. *anitratus* reported sensitive to cefotaxime had been grown from two tracheostomy site swabs (clinical specimens) taken seven and ten days prior to this screening sample. It was

not until nine months later that isolate 46 was grown from the throat swab of a 59-year-old patient (patient 95) on his second day in ITU following a pneumonectomy for infiltrating adenocarcinoma of the lung.

Seventeen of the 31 isolates each had one ß-lactamase with a pI value between 8.0 and 8.9. Isolates 3, 46, 114 and 130 have already been mentioned. Isolate 106 was resistant to gentamicin; otherwise its antibiogram and those of the other twelve isolates were unremarkable. Isolates 105,107, 115 and 120 (all *Acinetobacter calcoaceticus* var. *anitratus*) each had a similar plasmid of *c.*10 kb and an enzyme of pI value 8.5, 8.0, 8.7 and 8.9 respectively. Isolate 120 differed from the other three isolates in being resistant to gentamicin. Otherwise the antibiograms of the four isolates were very similar and remarkably sensitive. There was no apparent epidemiological connection. Isolates 105 and 107 had been isolated from throat swabs taken from a 68-year-old patient (patient 23) on her first and third days in ITU following cardiac surgery. Isolate 115 had been grown almost four weeks later from a throat swab taken from a 54-year-old patient (patient 99) on his first day in ITU following a hemicolectomy and transplant nephrectomy. Isolate 120 had been obtained (almost four weeks after strain 115) from a rectal swab taken from a 62-year-old patient (patient 67) on his fifth day in ITU following a coronary artery bypass graft.

Isolates 191 (from patient 19) and 137 (from patient 81) each had two ß-lactamases. Those of isolate 191 were of pI values 5.6 and 8.8 but its antibiogram did not suggest the presence of a TEM-type ß-lactamase in that it was sensitive to carbenicillin, ticarcillin and piperacillin and it did not contain plasmids. Isolate 137 was positive in the Etest for ESBL production and is discussed in Chapter 4.

Isolates 13, 18 and 127 had a ß-lactamase of pI 7.8; isolates 111 and 196 had a ß-lactamase of pI 7.7 and isolates 112 and 132 had a ß-lactamase of pI 7.9. Isolate 18 (from patient 30) as previously mentioned was resistant to carbenicillin and ticarcillin and borderline sensitive to piperacillin. It was resistant to ceftazidime and

aztreonam as well as cefotaxime. It had a good zone of inhibition to ticarcillin/clavulanic acid but had no zone of inhibition to amoxycillin/clavulanic acid. It was resistant to cefoxitin. It contained nine plasmids. As it had a ß-lactamase of 7.7 there is the possibility of an SHV-type enzyme. Isolate 132 from patient 81 was resistant to gentamicin and had no plasmids. However, like isolate 137, it was positive in the Etest for ESBL detection and is discussed in Chapter 4. Although the other five isolates had ß-lactamases of 7.7 or 7.8 and four of them contained plasmids, their antibiograms were not typical of the presence of an SHV-type enzyme as they showed sensitivity to carbenicillin, ticarcillin and piperacillin.

Isolates 111, 112 and 114 (all *Acinetobacter calcoaceticus* var. *anitratus*) each contained two similar plasmids of *c.*16 kb and 68 kb and had been isolated from rectal swabs from 53-year-old patient (patient 4), taken on the third, sixth and eighth days of his ITU admission following a road traffic accident. Isolates 111 and 112 had enzymes of similar pI (7.8 and 7.9) and almost identical antibiograms. Isolate 114 had a ß-lactamase of pI 8.7. Its antibiogram differed from that of isolates 111 and 112 in showing reduced sensitivity to amoxycillin/clavulanic acid and piperacillin.

Isolates 132 and 137 were positive in the Etest for ESBL production. Plasmids were not detected in isolate 132 (*Acinetobacter calcoaceticus* var. *anitratus*) which had a ß-lactamase of pI 7.9. In the Etest its ceftazidime MIC was > 32 mg/L whereas its ceftazidime/clavulanic acid MIC was 0.38 mg /L. It had no zone of inhibition to ampicillin but gave a substantial zone (26 mm) to amoxycillin/clavulanic acid. It showed large inhibition zones to carbenicillin (26 mm), ticarcillin (27 mm), ticarcillin/clavulanic acid (30 mm), piperacillin (28 mm) and piperacillin/tazobactam (36 mm). Isolate 132 was sensitive to ciprofloxacin and resistant to gentamicin.

Isolate 137 (satisfactorily identified only to genus *Acinetobacter*) had three plasmids of *c.*5 kb, 6 kb and 51 kb and two ß-lactamases of pIs 7.9 and 8.6. In the

Etest for ESBL detection its ceftazidime MIC, like that of isolate 132, was > 32 mg/L while its ceftazidime/clavulanic acid MIC was < 0.125 mg/L. This isolate was resistant to ampicillin (no zone of inhibition) but had a large zone to amoxycillin/clavulanic acid (25 mm). It showed large inhibition zones to carbenicillin (25 mm) and ticarcillin (24 mm) but gave a substantially larger zone (36 mm) to ticarcillin/clavulanic acid. It had an inhibition zone to piperacillin of 23 mm and only a slightly larger zone to piperacillin/tazobactam (25 mm). It showed reduced sensitivity to ciprofloxacin and was resistant to gentamicin.

3.7.2 *Citrobacter freundii*

Ten strains of *Citrobacter freundii* were isolated. All strains were sensitive to the carbapenems and mecillinam and all were resistant to cefoxitin. All were resistant to ceftazidime and all but one were resistant to aztreonam. ESBL production was non-determinable in the Etest for all isolates.

Isolate 4 showed borderline susceptibility to temocillin but was resistant to all other ß-lactams tested except the carbapenems and mecillinam. The isolate had a ß-lactamase of pI 8.3 and plasmids were not detected. Isolate 5 was very similar except that it was resistant to temocillin. Isolates 4 and 5 had been grown from rectal swabs taken from patient 56, aged 61 years, on his first and third days in ITU with respiratory and cardiac failure and possible adult respiratory distress syndrome superimposed on chronic obstructive airways disease. Antibiotic treatment before admission to ITU had not been recorded.

Isolate 9 was the most sensitive of the isolates of *Citrobacter freundii*. It was sensitive to carbenicillin, showed borderline susceptibility to ticarcillin and was sensitive to ticarcillin/clavulanic acid, amoxycillin/clavulanic acid, piperacillin/tazobactam, aztreonam and cefoxitin. It was resistant to ceftazidime (MIC >32 mg/L) and piperacillin. The isolate had a ß-lactamase of pI 8.3 and a plasmid of *c.* 125 kb. This isolate was grown from a rectal swab taken from patient

1, aged 62 years, on her eighth day in ITU following cardiac arrest in a medical ward. The patient had been found collapsed at home four weeks earlier and admitted to the medical ward with a deep vein thrombosis, pulmonary embolism, congestive cardiac failure and pneumonia. She had received amoxycillin and erythromycin before her ITU admission at which time SPEAR was started and amoxycillin stopped.

Isolate 97 was resistant to all the ß-lactams tested other than the carbapenems and mecillinam. It had a plasmid of *c.*8 kb and a ß-lactamase of pI 8.1. It was grown from a 61-year-old patient (patient 70) on her fifth day in ITU with respiratory and renal failure following a laparotomy for peritonitis. The patient had been transferred from another hospital.

Isolates 118, 119 and 122 showed reduced sensitivity or resistance to all the ß-lactams tested other than the carbapenems and mecillinam. These isolates were grown from rectal swabs taken from patient 87, aged 65 years, on her first, fourth and sixth days in ITU. The patient had been transferred from another hospital in septic shock following biliary bypass surgery. She had received perioperative cefuroxime, gentamicin and metronidazole. ß-Lactamase bands were detected as follows: pI 8.7 (isolate 118), pI 8.5 (isolate 119), pIs 7.9 and 8.7 (isolate 122).

Isolate 138 showed reduced sensitivity to cefotaxime (MIC 8 mg/L) and was resistant to all other ß-lactams tested other than the carbapenems and mecillinam. Resistance to piperacillin was overcome by tazobactam. This isolate contained six plasmids and had a ß-lactamase of pI 8.4. It had been grown from a rectal swab taken from patient 66, aged 59 years, on his third day in ITU with respiratory and renal failure.

Isolate 153 showed reduced sensitivity to piperacillin and was resistant to all other ß-lactams tested other than the carbapenems and mecillinam. It had a ß-lactamase of pI 8.6. The strain had been isolated from a rectal swab taken from a 66-

year-old patient (patient 38) on his fifth day in ITU with pancreatitis and respiratory acidosis.

Isolate 165 was resistant to all ß-lactams tested other than the carbapenems and mecillinam. It had a ß-lactamase of pI 8.4. The strain had been isolated from a rectal swab taken from patient 29, aged 74 years, on her ninth day in ITU with respiratory failure and an acute exacerbation of chronic obstructive airways disease.

3.7.3 *Enterobacter*

Fifty-one isolates belonging to the genus *Enterobacter* were studied. Forty-nine were identified as *Enterobacter cloacae* and two isolates (121 and 135) were identified satisfactorily to genus only. All strains had cefotaxime MICs equal to or > 32 mg/L and 46 strains had ceftazidime MICs > 32 mg/L. All isolates were negative in the Etest for ESBL production. All were sensitive to the carbapenems imipenem and meropenem and to mecillinam and ciprofloxacin. Eleven isolates were resistant to gentamicin.

Isolates 7 and 8 were resistant or showed reduced susceptibility to all the ß-lactams other than the carbapenems, mecillinam and piperacillin. They contained a similar plasmid of *c.*93 kb and had ß-lactamases of pI 8.3. They had been grown from rectal swabs taken from a 44-year-old patient (patient 93) on her first and third days in ITU. She had been admitted from the cardiothoracic unit as a ventilatory weaning problem following a tricuspid valve replacement and second mitral valve replacement five days earlier. Antibiotic therapy prior to her ITU transfer was not recorded. It is likely that isolates 7 and 8 had derepression of their chromosomally-mediated ß-lactamases.

Isolate 22 had two small plasmids and a ß-lactamase of pI 7.9. It was resistant or showed reduced susceptibility to all the ß-lactams other than the carbapenems, mecillinam and piperacillin. The organism had been grown from a rectal swab taken

from a 56-year-old patient (patient 76) on his first day in ITU following bowel resection and saphenous vein graft for a superior mesenteric artery thrombosis.

Isolate 26 had two plasmids of *c.*4 kb and 125 kb, a ß-lactamase of 7.7 and an antibiogram which showed resistance or reduced susceptibility to all the ß-lactams except the carbapenems and mecillinam. The isolate had been grown from the throat swab of a 63-year-old patient (patient 9) taken on the first day of her readmission to ITU from the cardiothoracic unit having had a cardiac arrest. She had already spent four days in ITU following a lobectomy seven days previously for a bronchogenic carcinoma. Cefuroxime had been given perioperatively and SPEAR had been given during her first 4-day ITU admission. Amoxycillin had been started the day before her readmission to ITU.

Isolates 33, 34 and 35 were sensitive to the carbapenems and mecillinam and showed borderline sensitive zones of inhibition with piperacillin. Otherwise they were resistant or showed reduced susceptibility to the other ß-lactams. These isolates did not contain plasmids. Isolates 33 and 34 had two ß-lactamase bands with pIs of 8.1 and 8.7. Isolate 35 had ß-lactamase bands with pIs of 8.0 and 8.4 on a different gel. The organisms had been isolated from rectal swabs taken from a 73-year-old patient (patient 52) on his first, sixth and eighth days in ITU following repair of an abdominal aortic aneurysm, renal failure and clinical sepsis. The patient had been admitted to ITU from another hospital where amoxycillin had been given for bronchitis.

Isolates 36 and 37 each had a plasmid of *c.*125 kb and a ß-lactamase of 8.1. They were resistant or showed reduced susceptibility to all the ß-lactams other than the carbapenems, mecillinam and temocillin. These isolates had been grown from rectal swabs taken from a 64-year-old patient (patient 16) on the first days of his second and third admissions respectively to ITU, fourteen days apart. The patient suffered from non-Hodgkin's lymphoma and had undergone splenectomy followed by an overnight stay in ITU ten weeks previously. His second ITU admission followed

resection of ischaemic bowel and his third followed breakdown of the anastomosis. He had been receiving long-term flucloxacillin before his splenectomy for an infected trephine biopsy wound. Unfortunately his history of antibiotic therapy outwith the ITU was not available. The resistance patterns of isolates 36 and 37 were probably caused by chromosomally-mediated ß-lactamase derepression unrelated to SPEAR.

Isolates 39 and 40 each had six plasmids, five of which (*c.*6 kb, 18 kb, 31 kb, 53 kb and 125 kb) were similar and each had one (*c.*11 kb in isolate 39) not observed in the other (*c.*102 kb in isolate 40). Isolate 39 had ß-lactamase bands of pIs 8.0 and 8.8 and isolate 40 had a ß-lactamase band of pI 8.3. These organisms had been isolated from two rectal swabs taken from a 59-year-old patient (patient 60) on his second day in ITU. He had been admitted for respiratory and cardiovascular monitoring following coronary artery grafts and mitral valve annuloplasty. Their antibiograms differed slightly. Isolate 39 showed borderline susceptibility to piperacillin, aztreonam and temocillin while isolate 40 showed reduced susceptibility to piperacillin and was resistant to aztreonam and temocillin. Both were susceptible to the carbapenems and mecillinam.

Isolates 43 and 47 each gave rise to multiple ß-lactamase bands with pIs ranging from 5.6 to 8.8. Isolate 43 contained two plasmids of *c.*5 kb and 77 kb. Isolate 47 had three plasmids of *c.*2 kb, 91 kb and 141 kb. The isolates were resistant or showed reduced susceptibility to all the ß-lactams other than the carbapenems and mecillinam. Isolate 43 had been grown from a faecal sample from a 48-year-old patient (patient 91) on the twelfth day of her admission to the ITU following surgery for small bowel obstruction and division of adhesions. Isolate 47 was obtained from the rectal swab of a 57-year-old patient (patient 75) taken on his first day in the ITU following debridement of widespread necrotizing fasciitis, four weeks after the previous patient's discharge.

Isolates 50 and 51 were grown from the same rectal swab of a 50-year-old patient (patient 41) on her second day in ITU after an aorta-superior mesenteric artery graft. She had been transferred from another hospital with gangrene of the superior mesenteric artery following cholecystectomy twelve days earlier. Isolate 50 had two plasmids of *c.*3 kb and 141 kb. Only the larger plasmid was detected in isolate 51. A ß-lactamase band of pI 7.6 was detected in both isolates which showed resistance or reduced sensitivity to all the ß-lactams other than the carbapenems, mecillinam and temocillin. Cefotaxime and metronidazole had been started prior to her ITU admission but other details of antibiotic therapy were not available. Resistance was possibly caused by chromosomally-mediated ß-lactamase derepression not related to SPEAR but perhaps to prior therapy the details of which were not available. The pI value of 7.6 and the presence of the large plasmid raises the possibility also of an SHV-type enzyme, either SHV-1 or an ESBL.

Isolate 52 was grown from the throat swab of a 63-year-old patient (patient 88) on his first day in ITU after transfer, collapsed and septic, from another hospital. A perforated duodenal ulcer had been oversewn eleven days earlier. A second laparotomy following his collapse nine days later revealed several gastric ulcers. Total gastrectomy and oesophago-jejunostomy were carried out. Isolate 52 contained a large plasmid of *c.*163 kb and had a ß-lactamase of pI 7.6. It was resistant to all the ß-lactams other than the carbapenems and mecillinam. Unfortunately the patient's antibiotic therapy prior to his ITU admission had not been recorded. Isolate 52 was not apparently related epidemiologically to isolates 50 and 51 although the patients' stays in the ITU overlapped.

Isolates 53 and 54 had been grown from rectal swabs taken from a 17-year-old patient (patient 5) on her first and third days in ITU after transfer from another hospital with nephrotic syndrome. Metronidazole had been given prior to her ITU admission. The isolates had a similar plasmid profile with multiple plasmids, the largest being *c.*88 kb and a ß-lactamase of pI 7.6. They were resistant to all the ß-

lactams other than the carbapenems and mecillinam. As with isolates 50 and 51 from patient 41 and isolate 52 from patient 88, resistance was possibly caused by chromosomally-mediated ß-lactamase derepression not related to SPEAR. The pI of 7.6 and the presence of the plasmids raises the possibility also of an SHV-type enzyme, either SHV-1 or an SHV-derived ESBL. Isolates 53 and 54 were not apparently related epidemiologically to isolates 50 and 51 or 52 and the plasmid profiles were quite different, although the ITU stays of patients 5, 41 and 88 overlapped.

Isolates 61 and 62 and 64, 65 and 66 were grown from rectal swabs taken from a 53-year-old patient (patient 89) on his second, fourth, eleventh, fourteenth and sixteenth days in ITU. He had been admitted with necrotizing fasciitis surrounding his sternal wound following a coronary artery bypass graft. Flucloxacillin had been started four days before admission to ITU. Plasmids were not detected in any of the strains. Isolates 61 and 62 each had ß-lactamases of pIs 6.8 and 7.5, isolates 64 and 65 each had a ß-lactamase of pI 7.6 and isolate 66 had ß-lactamases of pIs 4.9 and 7.9 All isolates were resistant to all the ß-lactams other than the carbapenems and mecillinam.

Isolates 77-81 were grown from throat and rectal swabs taken from a 57-year-old patient (patient 62) on her first and second days in ITU following a polyagastrectomy. The isolates had identical plasmids of *c.*4 kb, 13 kb and 181 kb. ß-Lactamase bands were detected as follows: pI 7.6 (isolate 77); pIs 7.6 and 8.0 (isolates 78, 79 and 81) and pIs 6.8, 7.6 and 8.0 (isolate 80). Isolates 78 and 80 were resistant to all the ß-lactams apart from the carbapenems and mecillinam. Isolates 77 and 79 were different. Although their cefotaxime MICs were also > 32 mg/L they showed susceptibility by disk diffusion to piperacillin, aztreonam and ceftazidime and the ceftazidime MIC for each isolate was 2 mg/L. Isolate 81 had a cefotaxime MIC of > 32 mg/L. It showed a reduced inhibition zone with piperacillin but was

susceptible by disk diffusion to aztreonam and ceftazidime. Its ceftazidime MIC was 3 mg/L. All five isolates were resistant to gentamicin.

Isolates 92 and 94 were grown from rectal swabs taken from a 61-year-old patient (patient 59) on his second and fourth days in ITU. He had been admitted in respiratory and renal failure from another hospital. An elective inguinal hernia repair twelve days earlier had been followed by intestinal obstruction, laparotomy for division of adhesions and pneumonia. The isolates each contained three plasmids of *c.*2 kb, 103 kb and 181 kb and each had ß-lactamases of pIs 7.1, 7.6 and 8.1. Their antibiograms showed reduced susceptibility to all the ß-lactams except the carbapenems and mecillinam.

Isolates 100, 101 and 102 were grown from rectal swabs taken from a 79-year-old patient (patient 51) on her first, second and fourth days in ITU with peritonitis and decreasing renal function following repair of an anastomosis. She had undergone a hemicolectomy twelve days earlier. Cefotaxime and metronidazole had been started at that time and stopped after four days. Ampicillin was then given for one day after which she was admitted to ITU and SPEAR started. The isolates each had three plasmids of *c.*4, 9 and 162 kb; an extra 34 kb plasmid was found in isolate 101. Beta-lactamase bands were detected with the following pI values: 8.1 (isolate 100), 7.7, 8.1 and 8.5 (isolate 102), 8.3 and 8.5 (isolate 102). Their antibiograms were almost identical and showed reduced susceptibility to all the ß-lactams except the carbapenems and mecillinam.

Isolate 116 carried three plasmids (*c.*5 kb, 11 kb and 214 kb) and had a ß-lactamase of pI 7.9. It was sensitive to the carbapenems, mecillinam, aztreonam, piperacillin and ceftazidime (MIC 3 mg/L in the Etest). It was resistant to cefoxitin. A borderline susceptible zone of inhibition was observed with ticarcillin whereas there was no inhibition zone with ticarcillin/clavulanic acid. The isolate was resistant to gentamicin. It had been grown from a rectal swab taken from a 74-year-

old patient (patient 61) on his fourth day in ITU with clinical sepsis following bilateral axillo-femoral grafts and repair of an aortic aneurysm.

Isolate 126 had been grown from a rectal swab taken from a 22-year-old patient (patient 40) on his third day in ITU following a second laparotomy to repair stabbing injuries. The isolate contained three plasmids of *c.*4 kb, 13 kb and 120 kb. Beta-lactamase bands of pIs 6.1, 6.5, 6.9 and 7.8 were detected. The isolate showed reduced susceptibility to all the ß-lactamases except the carbapenems and mecillinam.

Isolates 134, 135 (identified to genus only), 141 and 142 were resistant to all the ß-lactams except the carbapenems and mecillinam. They were resistant also to gentamicin. The isolates were grown from rectal swabs taken from a 51-year-old patient (patient 34) on her fifteenth and eighteenth days in ITU following surgery for the repair of a large hiatus hernia. Beta-lactamase bands of pI 7.7 (isolate 134) 7.8 (isolate 135) and 8.0 (isolates 141 and 142) were detected. Each strain had a large plasmid. Isolates 135 and 142 had identical plasmids of *c.*198 kb. Isolates 134 and 141, examined on a different agarose gel, had identical plasmids of *c.*165 kb. However it is possible that the different sizes represented the same plasmid because of the wide error margins already addressed.

Isolate 146 gave a borderline susceptible zone to temocillin but otherwise showed reduced susceptibility or resistance to all the ß-lactams except the carbapenems and mecillinam, as did isolates 147 and 148. These strains were isolated from a 69-year-old patient (patient 31) who had been transferred from another hospital with adult respiratory distress syndrome and sepsis following a hemicolectomy nine days earlier. Isolate 146 was grown from a rectal swab taken on her first day, and isolates 147 and 148 from throat and rectal swabs respectively, taken on her fifth day in ITU. A ß-lactamase of pI 8.0 was found in each of the isolates. Isolates 146 and 147 each had a large plasmid of *c.*120 kb on the same gel. Although the plasmid in

isolate 148 was found to be *c.*113 kb on a different gel it could have been the same plasmid because of the wide error margins already addressed.

Isolates 149 and 150 were obtained from a 26-year-old patient (patient 50) transferred to ITU from another hospital in septic shock secondary to peritonitis. The patient who suffered from Crohn's disease had had a large perianal abscess drained five weeks earlier followed by ischaemia of the colon, faecal peritonitis and a panproctocolectomy. Isolate 149 was grown from a throat swab and isolate 150 from a rectal swab taken on her third and twelfth days in ITU respectively. Both isolates had plasmids of *c.*6. 22 kb and 46 kb and another plasmid of *c.*171 kb was detected in isolate 150 only. A ß-lactamase of pI 8.6 was detected in both isolates whose antibiograms showed reduced susceptibility or resistance to all the ß-lactams apart from the carbapenems and mecillinam.

Isolate 161 was resistant to all the ß-lactams apart from the carbapenems. A ß-lactamase of pI 8.0 was observed. Plasmids were not detected. The isolate was grown from a rectal swab taken from a 37-year-old renal transplant patient (patient 14) on his fourth day in ITU with *Legionella pneumophila* pneumonia. He had started ampicillin and flucloxacillin the day before admission to ITU where flucloxacillin was stopped. Ampicillin, erythromycin and SPEAR (cefotaxime and SDD) were given for four days then ampicillin and cefotaxime were stopped and ciprofloxacin substituted. Treatment with SDD, erythromycin and ciprofloxacin was continued for eleven days.

Isolates 174, 178 and 184 were grown from rectal swabs taken from a 73-year-old patient (patient 24) on his first, sixth and eighth days in ITU following a possible respiratory arrest. Having sustained multiple injuries in a fall he had been admitted to an orthopaedic ward fifteen days before his ITU admission. Isolates 174 and 178 had two identical small plasmids on the same agarose gel. Isolate 184 had undergone electrophoresis on a different agarose gel. It had four small plasmids of a similar size to those of isolates 174 and 178. ß-Lactamase bands with the

following pIs were detected: 6.8 and 8.6 (isolate 174), 8.6 (isolate 178), 6.9 and 8.7 (isolate 184). Isolate 174 was resistant or showed reduced susceptibility to all the ß-lactams except the carbapenems, mecillinam and temocillin. Isolate 178 was sensitive to the carbapenems and showed borderline susceptibility to piperacillin/tazobactam. It was resistant or showed reduced susceptibility to all the other ß-lactams including mecillinam and temocillin. Isolate 184 was sensitive to the carbapenems, mecillinam, temocillin and piperacillin. Tazobactam did not enhance the activity of piperacillin as it seemed to do in the case of isolate 178.

Isolate 197 was sensitive to the carbapenems and mecillinam but was resistant or showed reduced sensitivity to all the other ß-lactams. It had been grown from a rectal swab taken from a 60-year-old patient (patient 22) on her first day in ITU after transfer from another hospital. She had developed respiratory failure following an endoscopic retrograde cholopancreatectomy. The isolate had a ß-lactamase of pI 8.6.

Isolate 199 was sensitive to the carbapenems and mecillinam but was resistant or showed reduced sensitivity to all the other ß-lactams tested. The organism had been grown from a rectal swab taken from a 73-year-old patient (patient 71) on her second day in ITU after drainage of a subphrenic abscess. She had undergone repair of an aortic aneurysm six weeks earlier. Isolate 199 had a ß-lactamase of 8.2. The patient received ampicillin as well as SPEAR in ITU. Unfortunately details of antibiotic therapy prior to her ITU admission were not recorded. Culture of the subphrenic pus had yielded a strain of *Enterobacter* resistant to cefotaxime, but the rest of the antibiogram was not recorded.

Isolate 121 was sensitive to piperacillin, aztreonam, mecillinam, temocillin and the carbapenems. It was resistant to cefotaxime (MIC > 32 mg/L), showed reduced sensitivity to ceftazidime (MIC 4 mg/L) and was resistant also to gentamicin. It was negative in the Etest for ESBL production. It had been grown from the rectal swab of a 62-year-old patient (patient 67) on his fifth day in ITU with respiratory failure

following a coronary artery bypass graft. It had a large plasmid (*c.*165 kb) and a ß-lactamase of pI 7.9.

3.7.4 *Escherichia coli*

Two strains of *Escherichia coli* were isolated. The Etest for ESBL detection was negative for both strains and cefotaxime resistance was not transferred. Isolate 76 showed slightly reduced sensitivity to cefotaxime (MIC 4 mg/l) but was sensitive to ceftazidime and aztreonam. It showed borderline susceptibility to temocillin and was sensitive to cefoxitin. Except for the carbapenems it was resistant to the other ß-lactams tested and resistant also to amoxycillin/clavulanic acid, ticarcillin/clavulanic acid and piperacillin/tazobactam. The isolate contained two plasmids of *c.*73 kb and 92 kb and had two ß-lactamases of pIs 5.4 and 7.4. This isolate had been grown from a rectal swab taken from a 60-year-old patient (patient 98) on her fifth day in ITU. She had suffered severe blood loss from a pathological fracture of her femur secondary to myeloma.

Isolate 200 showed resistance or reduced sensitivity to all the ß-lactams tested other than the carbapenems and mecillinam. The isolate contained three plasmids of *c.*5 kb, 11 kb and 56 kb and had one ß-lactamase of pI 8.5. This strain was isolated from a rectal swab taken from a patient (patient 15) on his second day in ITU after laparotomy for breakdown of a colonic anastomosis. He had undergone anterior resection ten days earlier for rectal carcinoma.

3.7.5 *Flavobacterium odoratum*

Two strains of *Flavobacterium odoratum* were isolated. ESBL production was not determinable by Etest in either strain. Isolates 89 and 90 were sensitive to piperacillin and cefoxitin by disk diffusion testing but resistant to all other ß-lactams tested including the carbapenems imipenem and meropenem. As expected for *Flavobacterium* species the isolates were resistant also to gentamicin. They were grown from throat swabs taken from a 70-year-old patient (patient 73) on his fourth

day in ITU after an oesophagectomy for carcinoma. Plasmids were not detected in either strain and a ß-lactamase of pI 7.8 was detected in isolate 89 only.

3.7.6 *Hafnia alvei*

Four strains of *Hafnia alvei* were isolated. All were sensitive to the carbapenems imipenem and meropenem. ESBL production was non-determinable in the Etest for all four strains. Isolate 75 had two plasmids of *c.*42 kb and 119 kb and two ß-lactamases with pI values of 7.4 and 7.8. The isolate was resistant to cefotaxime and ceftazidime but was sensitive by disk diffusion testing to cefoxitin. Except for the carbapenems and mecillinam it showed resistance or reduced sensitivity to the other ß-lactams including the ß-lactamase inhibitor combinations. The isolate had been grown from a throat swab taken from a 51-year-old patient (patient 86) on his first day in ITU with respiratory failure. The patient had disseminated lymphoma and diabetes and had been transferred from a medical ward.

Isolates 167, 168 and 169 were all grown from a 64-year-old patient (patient 43). Isolates 168 and 169 were grown from a throat swab and rectal swab respectively taken from the patient on her first day in ITU with renal failure following a hemicolectomy four days earlier at another hospital. Isolate 167 was grown from a rectal swab taken on her second day in ITU. A ß-lactamase of 7.8 was detected in all isolates. Six plasmids (*c.*5 kb, 6 kb, 11 kb, 14 kb, 51 kb and 59 kb) were observed in isolate 167: plasmids similar to the first four of these were detected also in isolate 168 and plasmids similar to the first five in isolate 169. All three isolates were resistant to cefotaxime and ceftazidime. Isolate 168 showed only borderline sensitivity to aztreonam and was resistant to carbenicillin, ticarcillin piperacillin, piperacillin/tazobactam and temocillin. Isolate 167 was sensitive to temocillin and aztreonam but showed reduced sensitivity to carbenicillin, ticarcillin, piperacillin and piperacillin/tazobactam. Isolate 169 was resistant to carbenicillin. It showed borderline susceptibility to temocillin and reduced sensitivity to ticarcillin,

piperacillin and piperacillin/tazobactam but was sensitive to aztreonam. All three isolates were sensitive to cefoxitin and resistant to amoxycillin/clavulanic acid.

3.7.7 *Klebsiella pneumoniae*, ESBL production and transfer of ceftazidime resistance

Two strains of *Klebsiella pneumoniae* (133 and 140) were isolated. Although both were sensitive to cefotaxime, isolate 140 was resistant to ceftazidime and isolate 133 showed only borderline sensitivity by disk diffusion testing. Both were resistant to gentamicin. For these reasons they were suspected of harbouring an ESBL and were retained for further investigation. The Etest for ESBL detection was positive for both isolates (Appendix IV). They had been grown from rectal swabs taken from a 29-year-old patient (patient 81) on her sixth and eleventh days respectively in ITU with respiratory failure. Hodgkin's disease had been diagnosed four years earlier and she had undergone a bone marrow transplant six weeks prior to her ITU admission. Ceftazidime had been started six days before she was admitted to the ITU and was continued along with SDD throughout her ITU stay. The patient had also received tobramycin.

Plasmid analysis of isolates 133 and 140 had originally been carried out on different agarose gels. Isolate 133 had four plasmid bands of *c.*5 kb, 10 kb, 54 kb and 182 kb and two ß-lactamases of pIs 5.4 and 7.6 suggesting a TEM-type and SHV-type of enzyme respectively. The MIC of isolate 133 for ceftazidime was 8 mg/L and that for cefotaxime was < 0.5 mg/L. By disk diffusion testing isolate 133 was sensitive to aztreonam and piperacillin/tazobactam but was resistant to amoxycillin/clavulanic acid, carbenicillin, ticarcillin, ticarcillin/clavulanic acid and piperacillin. It was sensitive also to cefoxitin, mecillinam, and the carbapenems and resistant to gentamicin. Isolate 140 also contained four plasmids of *c.*5 kb, 12 kb, 48 kb and 168 kb and had two ß-lactamases of pIs 5.3 and 7.6 suggesting a TEM-type and SHV-type of enzyme respectively. Its antibiogram was similar to that of isolate

133 except that its ceftazidime MIC was 32 mg/L. Although the isolates were resistant to amoxycillin/clavulanic acid there was synergy between disks of amoxycillin/clavulanic acid and ceftazidime.

In the additional transconjugation studies using ceftazidime rather than cefotaxime along with rifampicin for counterselection, transconjugants were obtained from both isolates at a ceftazidime concentration of 1 mg/L but not at the higher ceftazidime concentration of 4 mg/L. Transconjugants from isolate 140 were shown to have a large plasmid of *c.*214 kb but plasmids were not detected in transconjugants from isolate 133 (Figures 21 and 22). However transconjugants from both donor isolates 133 and 140 were shown to have the TEM-type ß-lactamase as well as the ß-lactamase of pI 8.4 of the recipient *Escherichia coli* J62-2, but not the SHV-type enzyme of the donor *Klebsiella* organisms. Resistance to ceftazidime in these isolates was almost certainly caused by an ESBL of TEM-type. The enzymes of pI 7.6 in both isolates were likely to be SHV-1 enzymes. Most *Klebsiella pneumoniae* strains have chromosomally mediated SHV-1 ß-lactamases [98] which are constitutive and produced usually at low levels.

Isolates 133 and 140 were obtained from samples taken on the patient's sixth and eleventh days in ITU and had ceftazidime MICs of 8 and > 32 mg/L respectively. Both gave positive results in the Etest for ESBL production. Transfer of ceftazidime resistance in both isolates was successful using the lower ceftazidime concentration of 1 mg/L for counterselection, but not with the higher concentration of 4 mg/L. Cefotaxime MICs for isolates 133 and 140 were 0.06 and 0.25 mg/L respectively.

3.7.8 *Morganella morganii*

Nine strains of *Morganella morganii* were isolated from six patients. Plasmids were detected in four of these isolates and ß-lactamase bands were observed in all of them. Isoelectric points ranged from 5.4 to 7.9. All of the isolates were sensitive to the carbapenems imipenem and meropenem and all were resistant to ampicillin and

amoxycillin/clavulanic acid. Resistance to cefotaxime was not transferred by any of the isolates of *Morganella morganii* and ESBL production was not determinable by Etest.

Isolate 10 had a plasmid of *c.*71 kb and a ß-lactamase of pI 7.4 which aligned with OXA-1 on the same gel. However the isolate was sensitive to carbenicillin and had a borderline inhibition zone with ticarcillin. It was resistant to cefotaxime, ceftazidime and piperacillin but was sensitive to piperacillin/tazobactam, cefoxitin and aztreonam. The isolate had been grown from a rectal swab taken from a patient (patient 1) on her tenth day in ITU following cardiac arrest in a medical ward. The patient had been found collapsed at home four weeks earlier and had been admitted with a deep vein thrombosis, pulmonary embolism, congestive cardiac failure and pneumonia. She had received amoxycillin and erythromycin before her ITU admission.

Isolate 16 showed reduced sensitivity to cefotaxime and was resistant to ceftazidime. It was sensitive by disk diffusion testing to aztreonam. It gave borderline sensitive inhibition zones with ticarcillin, piperacillin and cefoxitin and showed reduced sensitivity to carbenicillin. One ß-lactamase of pI 7.9 was detected. This isolate had been grown from a throat swab taken from a patient (patient 33) on his first day in ITU with renal failure following an elective aorto-femoral bypass three days earlier at another hospital. *Morganella morganii* was isolated also from clinical samples.

Isolate 23 was grown from a rectal swab taken from a 59-year-old patient (patient 82) on her first day in ITU after desloughing of an old Caesarean section wound. The isolate was resistant to all the ß-lactams tested other than the carbapenems. It had three plasmids (*c.*6 kb, 12 kb and 47 kb) and two ß-lactamases of pIs 6.5 and 7.8. *Morganella morganii* resistant to cefotaxime had been isolated also from clinical specimens from this patient.

Isolate 24 was grown from a rectal swab taken two days later from the same patient (patient 82) as isolate 23. It had a plasmid profile identical to that of isolate 23, but had only one ß-lactamase of pI 7.7 and a slightly different antibiogram. It was sensitive to ticarcillin (borderline inhibition zone), temocillin, aztreonam, and cefoxitin. It showed reduced sensitivity to cefotaxime and was resistant to ceftazidime. By disk diffusion testing it showed reduced sensitivity to carbenicillin and was resistant to piperacillin but gave a good zone of inhibition to piperacillin/tazobactam.

Isolate 25 was grown from a rectal swab taken on the first day of the above patient's readmission to ITU fourteen days later following closure of a ventral hernia. This isolate had two plasmids (*c.*6 kb and 15 kb) and ß-lactamases of pIs 6.5 and 7.9. It was sensitive to carbenicillin and ticarcillin (both borderline inhibition zones), temocillin, aztreonam, cefoxitin and gentamicin. It showed reduced sensitivity to cefotaxime and was resistant to ceftazidime and piperacillin but sensitive to piperacillin/tazobactam.

Isolate 57 had ß-lactamases of pIs 5.4 and 6.8 and was resistant or showed reduced sensitivity to all the ß-lactams tested other than the carbapenems. The ß-lactamase band of 5.4 was suggestive of a TEM-type enzyme although plasmids were not detected. This isolate was grown from a rectal swab taken from a patient (patient 42) on his fifteenth day in ITU after a second laparotomy for cholecystectomy and drainage of a necrotic pancreas. *Morganella morganii* had been isolated from multiple clinical specimens taken two days earlier. The patient had received cefotaxime for the first three days of his ITU stay.

Isolate 58 was obtained from the same patient (patient 42) as isolate 57. It was grown from a rectal swab taken on his twentieth day in ITU. This isolate had only one ß-lactamase of pI 6.8 and was sensitive by disk diffusion to carbenicillin, ticarcillin and piperacillin. Like isolate 57 it showed reduced sensitivity to cefotaxime and was resistant to ceftazidime.

Isolate 113 was resistant to cefotaxime and ceftazidime but sensitive to aztreonam. It gave a substantial inhibition zone to carbenicillin and a borderline sensitive zone to ticarcillin. It showed reduced sensitivity to piperacillin but was sensitive to piperacillin/tazobactam and cefoxitin. Three ß-lactamase bands of pIs 6.5, 7.3 and 7.9 were observed. The isolate was grown from a rectal swab taken from a patient (patient 68) on his eighth day in ITU after transfer from another hospital. He was suffering from an acute exacerbation of chronic obstructive airways disease.

Isolate 157 was resistant to ceftazidime (MIC > 32 mg/l) although it gave a sensitive inhibition zone by disk testing. The isolate showed reduced sensitivity to cefotaxime and piperacillin but was sensitive to cefoxitin, carbenicillin, ticarcillin and aztreonam. A ß-lactamase band of pI 6.8 was detected. The isolate was grown from a rectal swab taken from a patient (patient 21) on his third day in ITU following laparotomy for peritonitis. He had been given perioperative metronidazole and gentamicin. On admission to ITU he received systemic tobramycin as well as SPEAR. The patient had spent two days in ITU four weeks earlier following hemicolectomy and splenectomy.

3.7.9 *Pseudomonas*

In the present study 57 pseudomonads were isolated. Of these, 50 were identified as *Pseudomonas aeruginosa*, one as *Pseudomonas fluorescens* and six isolates were satisfactorily identified to genus only. Cefotaxime MICs were 16 mg/L (three isolates), 32 mg/L (thirteen isolates) and > 32 mg/L (forty-one isolates). Plasmids were detected in only two isolates, 38 and 117, both *Pseudomonas aeruginosa*. ß-Lactamases were detected in 34 of the 57 pseudomonad isolates.

Isolate 38 had a plasmid of *c.*43 kb and ß-lactamase bands representing pI values of 7.9 and 8.8 were detected. Only one band occurred on each of two gels, the higher pI being recorded on the more accurate gel. The antibiotic sensitivity pattern

for the organism was unremarkable. These findings imply the basal production of a chromosomally-mediated enzyme.

Isolate 117 had plasmids of *c.*7 kb and 40 kb and a ß-lactamase of pI 7.9. There was complete resistance by disk diffusion to the third-generation cephalosporins cefotaxime and ceftazidime, the carboxypenicillins carbenicillin and ticarcillin, the ureidopenicillins azlocillin and piperacillin and the monobactam aztreonam. There was a minimal zone of inhibition with ticarcillin/clavulanic acid but none with piperacillin/tazobactam.

Isolate 27 had an antibiogram identical to strain 117 and a ß-lactamase which reacted immediately to nitrocefin spot-testing and banded strongly at pI 7.9. Plasmids were not detected in this isolate, otherwise all the comments applied to isolate 117 are pertinent to isolate 27.

Isolates 48, 49 and 103 had similar antibiograms to isolates 27 and 117. Beta-lactamases of pIs 8.2 and 8.7 were found in isolates 48 and 103 respectively; none was detected in isolate 49 on three different gels.

Isolates 55, 93 and 103 showed resistance to carbenicillin, azlocillin and ticarcillin by disk diffusion. Isolate 55 had a ß-lactamase of pI 7.8 which was presumably a chromosomal type. Isolates 55 and 103 had enzymes of pI 8.1 and 8.7 respectively, presumably chromosomal types.

Isolate 56 was resistant to carbenicillin and ticarcillin and to imipenem as well as meropenem. However it was sensitive to azlocillin, piperacillin, ceftazidime and ciprofloxacin. A ß-lactamase of pI 8.2, presumably chromosomally-mediated, was detected.

Isolates 60, 71, 72, 73, 83, 84 and 85 had borderline or slightly reduced sensitivities to carbenicillin but were otherwise unremarkable and ß-lactamases were not detected. Isolate 63 was resistant to carbenicillin and had a ß-lactamase of 7.5 which could possibly have indicated an OXA-type enzyme.

Isolates 139 and 163 were resistant to carbenicillin and strain 163 also showed reduced sensitivity to ciprofloxacin. ß-Lactamases were not detected in isolate 139 but ß-lactamase bands of pI 7.4 and 7.9 were detected in isolate 163. It is possible that the band of pI 7.4 represented an OXA-type enzyme and that of 7.9 a chromosomal enzyme.

Isolates 144, 152 and 162 showed reduced sensitivity to carbenicillin but were otherwise unremarkable. All three had high pI enzymes probably of chromosomal type.

Isolates 182, 183, 190 and 198 showed reduced sensitivity to carbenicillin but were sensitive to ticarcillin by disk diffusion testing and clavulanic acid did not particularly enhance the ticarcillin zones. ß-lactamases were not detected in isolates 190 and 198. Isolate 182 had an enzyme of pI 7.7. Isolate 183 had enzymes of 7.4 and 7.9.

Isolates 67, 68, 69 and 70 were identified to genus only. Isolates 67 and 68 had reduced sensitivities to carbenicillin but were sensitive to ticarcillin. Clavulanic acid seemed to have some effect in that larger zones of inhibition were exhibited with timentin (ticarcillin/clavulanic acid) than with ticarcillin alone and both isolates showed zones of inhibition around amoxycillin/clavulanic acid. However ß-lactamases were not detected.

Isolates 69 and 70 were resistant to carbenicillin and ticarcillin. Clavulanic acid seemed to counteract the latter resistance suggesting the possible involvement of a plasmid-mediated ß-lactamase. Strain 69 had a ß-lactamase of pI 7.6 but ß-lactamase bands were not detected in isolate 70.

Isolate 95, *Pseudomonas fluorescens*, was resistant to carbenicillin and ticarcillin. The activity of the latter was unaffected by clavulanic acid. A ß-lactamase of pI 8.3, presumably of chromosomal type, was detected.

Isolates 17, 19, 20, 42 and 151 had ceftazidime MICs > 32 mg/L although all isolates gave inhibition zones clearly denoting susceptibility to ceftazidime as well

as piperacillin and aztreonam on disk diffusion testing. ESBL production was not determinable by Etest on the five isolates. B-Lactamases of pIs 8.2, 8.1, 8.3 and 8.4, probably denoting chromosomal types, were found in isolates 19, 20, 42 and 151 respectively. None was detected in isolate 17. Plasmids were not detected in any of the five isolates.

Isolate 17 was grown from a rectal swab taken from a 58-year-old patient on his first day in ITU with renal failure following an aorto-femoral bypass at another hospital. Isolates 19 and 20 were grown from a rectal swab taken from an 83-year-old patient on her first day in ITU following a cataract extraction and lens implant. Details of antibiotic therapy prior to ITU admission were not available for either of these patients.

Isolate 42 was grown from a rectal swab of a 73-year-old patient (patient 64) taken on his fourth day in ITU with renal failure following cholecystectomy at another hospital. A rectal swab taken on the patient's first day in ITU yielded isolate 41 with a ceftazidime MIC of 8 mg/L, an unremarkable antibiogram, a ß-lactamase of pI 8.3 (presumably chromosomal) and no plasmids.

Isolate 151 was grown from a rectal swab of a 67-year-old patient (patient 41) taken on his first day in ITU following cardiac septal rupture. A rectal swab taken from the same patient ten days later yielded isolate 152 with a ceftazidime MIC of 4 mg/L, an unremarkable antibiogram, ß-lactamase bands of 8.2 and 8.4 and no plasmids. Isolate 151 which was resistant to ceftazidime was grown from a sample taken on the patient's first day in ITU while isolate 152 which was sensitive to ceftazidime was isolated ten days later.

The remaining 21 pseudomonads had unremarkable antibiograms. ß-Lactamases were detected in 11 of these isolates. Nine had high pI enzymes of 7.9 and above, which were probably chromosomally determined and one had a ß-lactamase of pI 7.7 probably also of chromosomal type. One had two bands of pI 7.4 and 7.9 respectively. Its cefotaxime MIC was > 32 mg/L but otherwise the antibiogram

indicated a generally sensitive strain. It is likely that the enzymes of this isolate were present in small amounts as they could be detected only after disk induction with cefoxitin.

In summary the most noteworthy pseudomonads were isolates 117 and 27; isolate 49 which had a similar antibiogram but no detectable ß-lactamase band and isolate 56 which was resistant to both carbapenems imipenem and meropenem.

Isolate 117 had been grown from the rectal swab of a patient (patient 61) on his fifteenth day in ITU following bilateral axillo-femoral grafts. Clinically he had sepsis and acute renal failure. He had undergone SPEAR and had received in addition flucloxacillin (for a bacteriologically confirmed *Staphylococcus aureus* infection) and parenteral tobramycin.

Isolate 27 had been grown from the rectal swab of a patient (patient 58) with late-onset asthma on her eighth day in ITU with a severe chest infection. She had undergone SPEAR, except that cefotaxime was used as therapy rather than prophylaxis in her case, doses and length of course being increased appropriately. The patient had also received erythromycin and rifampicin. Isolates 27 and 117 were obtained more than a year apart.

Isolate 49 had been grown from the rectal swab of a patient (patient 28) on his sixteenth day in ITU following a road traffic accident in which he had sustained multiple injuries. He underwent SPEAR, cefotaxime being continued for five days. Strain 49 was isolated ten days after cefotaxime had been stopped. Isolates 49 and 27 were obtained more than four months apart.

Isolate 56 had been grown from the throat swab of a patient (patient 45) on the first day of his admission to ITU. He had been transferred from another hospital for management of sepsis, renal failure and a left pyopneumothorax following a left hernia repair. Unfortunately no details of prior antibiotic therapy had been recorded. Isolates 56 and 49 were obtained from samples taken over a month apart

3.7.10 *Stenotrophomonas maltophilia*

Seven isolates of *Stenotrophomonas maltophilia* were investigated. Isolates 28, 29, 30, 31 and 32 were grown from rectal swabs from the same patient (patient 36) taken on her first (isolates 28 & 29), third, sixth and eighth days in ITU respectively. The patient was 75 years old, had a ruptured diverticulum, faecal peritonitis and respiratory failure. Isolates 28, 30, 31 and 32 had cefotaxime MICs > 32 mg/L and were resistant to all the antibiotics tested by disk diffusion other than ciprofloxacin and ticarcillin/clavulanic acid. Isolate 29 differed slightly in showing reduced sensitivity to ciprofloxacin and only a borderline zone of inhibition to ticarcillin/clavulanic acid. Only weak ß-lactamase activity was detected in nitrocefin spot-testing of all five cell extracts even after disk induction with cefoxitin. On isoelectric focusing a ß-lactamase of pI 6.1 was detected in all five isolates.

Isolate 99 was grown from the throat swab of a 79-year-old patient (patient 51) on the second day of her ITU admission following repair of a colonic anastomosis, peritonitis and deteriorating renal function. The antibiogram of this isolate was similar to that of isolates 28 and 30-32, but ß-lactamases were not detected. There was no temporal or epidemiological association between isolate 99 and the other isolates.

Isolate 45 was grown from the throat swab of a 59-year-old patient (patient 95) on his second day in ITU following a pneumonectomy for an infiltrating adenocarcinoma of the lung. It was apparently more sensitive *in vitro* than the strains isolated from the other two patients, showing large zones of inhibition to ciprofloxacin, ceftazidime, ticarcillin/clavulanic acid and piperacillin/tazobactam. It showed susceptibility also to carbenicillin, ticarcillin and azlocillin and borderline sensitivity to piperacillin. Three small plasmids were detected in this strain, but ß-lactamases were not demonstrated. Again there was no temporal or epidemiological link between isolate 45 and the other isolates.

Chapter 4 Discussion

4.1 Aims of the study

The aims of the study were to investigate the cefotaxime resistance mechanisms of 175 AGNB isolated from surveillance flora of ITU patients undergoing SPEAR and to assess the implications of such resistance for the continuation of SPEAR. This work was particularly concerned with the incidence of two mechanisms of cefotaxime resistance in AGNB with the potential for dissemination in the ITU as a result of the widespread use of cefotaxime as an integral part of SPEAR: chromosomal ß-lactamase derepression in the ß-lactamase-inducible species such as *Citrobacter freundii*, *Morganella morganii*, *Pseudomonas aeruginosa* and *Enterobacter* species and plasmid-mediated ESBL production, more common in *Klebsiella pneumoniae* and *Escherichia coli*, but found also in many other AGNB including the ß-lactamase-inducible species. Findings of such resistance in surveillance flora might be a portent of serious clinical resistance problems to come.

4.2 Plasmid analysis

4.2.1 Agarose gel size

Every isolate was screened for plasmids on at least one, and often multiple, medium-length (14 cm) gels and at least one long (25 cm) gel. More plasmids were detected on medium than on long gels in 54 of the 78 isolates which contained plasmids. The same number of plasmids was detected on both medium and long gels in 19 isolates and more plasmids were detected on long gels than on medium gels in only five isolates. Twenty-seven plasmids > 100 kb were detected on medium but not on long gels and only one large plasmid (171 kb) was detected on a long gel but not on a medium gel. In this study sensitivity of horizontal agarose gel electrophoresis was not improved by using the longer gel.

4.2.2 Plasmid size

It is appreciated that only an estimate of plasmid size was obtained in the present study. Agarose gel electrophoresis was not originally presented as a means for precise plasmid characterization [218] and other investigators [221] have also acknowledged the limitations of the method, because the logarithm/logarithm of plasmid size versus mobility is not a straight line over all values. In the present study the widest error margin occurred around the 38 kb size (section 3.3). Another group [235] claimed greater accuracy for all plasmid sizes in an entire range from 1.76 MDa (2.7 kb) to 312 MDa (491 kb) using a more complex multiple regression analysis. However throughout the present work the consistency of the experimental technique was reflected in the proximity of the r^2 statistic to '1'. If the results of plasmid screening and transconjugation studies had suggested the presence of one or more epidemic plasmids, then more accurate characterization would have been carried out using restriction endonuclease analysis of appropriately purified cell lysates [236].

4.3 Transconjugation experiments

Plasmids were detected in 78 of 175 isolates. In case any plasmids had not been detected by agarose gel electrophoresis and in case transfer of cefotaxime resistance might occur without plasmid transfer, transconjugation experiments were carried out on all 175 isolates whether or not plasmid bands had been demonstrated. Transferable resistance to cefotaxime was not detected in any isolate in this study using standard broth mating methods at the optimal growth temperatures of the organisms. Transferable resistance to ceftazidime was demonstrated in two isolates which were sensitive to cefotaxime but resistant to ceftazidime. It might be argued that some potential plasmid transfers were not expedited by the liquid mating techniques used in this study and therefore that some plasmid-mediated ESBL

production was not detected. Bradley and colleagues [237], studying plasmid transfers between strains of *Escherichia coli* in 1980, showed that surface (plate) mating was sometimes more efficient than liquid mating, especially in strains with rigid pili. Also some plasmids are temperature-sensitive [237] and their transfer might not have been detected in the present work as only those organisms which grew better at 30° C were "mated" at that temperature. The phylogenetic origins of the mating pairs must also be considered. The recipient organism was always *Escherichia coli* whereas the potential donors were of various genera, usually not *Escherichia coli*. Platt and Sommerville [238] suggested that an intergeneric barrier might limit genetic transfer between strains of different genera. Falkow and colleagues [139] suggested that the divergent structure of the genetic material affected chromosomal pairing and integration. However they did go on to describe gene transfer between two strains of different genera with divergent DNA base ratios.

4.4 Preparation of cell lysates

Slime production by mucoid organisms was mentioned as a problem in the preparation of cell lysates. In order to overcome this problem Hibbert-Rogers and colleagues [239] have since reported the use of snail acetone powder in the digestion of bacterial cell wall polysaccharides prior to DNA extraction procedures.

4.5 Isoelectric focusing, antibiogram and prediction of ß-lactamase type

Isoelectric focusing was carried out on all SPEAR isolates. As there are many well-established relationships between ß-lactamase activity and antibiogram [240] the isolates were screened also for susceptibility to a battery of ß-lactam antibiotics chosen to facilitate the recognition of the resistance patterns associated with certain ß-lactamase types. For example, isolate 119 was identified as *Citrobacter freundii*, one of the species known to have inducible chromosomal ß-lactamases. It was

resistant to cefoxitin, cefotaxime, ceftazidime, piperacillin and amoxycillin/clavulanic acid. It had a ß-lactamase of pI 8.5, plasmids were not detected and cefotaxime resistance was not transferred. It therefore seemed reasonable to infer that its resistance to cefotaxime was mediated by stable derepression of a chromosomal AmpC enzyme. However prediction of ß-lactamase type using isoelectric focusing and antibiogram has limits. If hypothetically such an isolate of *Citrobacter freundii* also had a ß-lactamase of pI 7.6 it might mean that a stably derepressed organism also produced a plasmid-mediated SHV-type ESBL or simply a parental SHV-1 enzyme. In such a situation the antibiogram would not be discriminatory and even a DNA probe would not distinguish between the latter two enzymes, but the appropriate genetic experiments might detect the transfer of genetic material. For this reason transconjugation experiments were performed on all SPEAR isolates in the present study in order to look for transfer of cefotaxime resistance. A further difficulty arises when the range of pIs quoted for the chromosomal ß-lactamases of a ß-lactamase-inducible species overlaps with the range of pIs for possible plasmid-mediated enzymes. This occurred with a number of the *Enterobacter* isolates and is discussed in detail below. In such cases the identity of the ß-lactamase cannot be inferred from the antibiogram. Prediction of ß-lactamase type using isoelectric focusing and antibiogram is of limited value also for organisms such as *Acinetobacter* species in which relationships between antibiogram and ß-lactamase profile are poorly established [240].

4.6 Chromosomal ß-lactamases, their mode of production and contribution to resistance

Chromosomal ß-lactamases are almost ubiquitous in enterobacteria except for salmonellae but vary greatly in amount, mode of production and therefore in their contribution to resistance [155,173]. Some species have molecular class A types but a greater number have molecular class C enzymes of Bush-Jacoby-Medeiros group

1, also known as AmpC ß-lactamases [169] whose expression may be inducible, high-level constitutive or low-level constitutive, according to the species and the strain. Chromosomal ß-lactamases are ubiquitous also in non-fermenting AGNB and as in enterobacteria are variable in amount and significance. The enzymes of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* have been studied extensively and their roles in resistance are well established; those of *Acinetobacter* and *Flavobacterium* species are less well understood according to Livermore in his review [170]. Certain species of AGNB, including *Pseudomonas aeruginosa*, *Enterobacter*, *Citrobacter* and *Morganella morganii*, have inducible expression of AmpC enzymes and show greater potential for resistance. ß-Lactamase induction has been the subject of confusion but is simply the transient elevation in ß-lactamase synthesis that occurs when a ß-lactam drug is present. Enzyme synthesis returns to a low level when the inducer is removed [241]. Individual ß-lactam antibiotics differ in their inducer power and lability (susceptibility to hydrolysis). First-generation cephalosporins induce AmpC enzymes strongly and are labile to hydrolysis. Consequently they cause their own destruction and lack activity against ß-lactamase inducible species [241]. Ampicillin shows this behaviour for most species except *Citrobacter freundii* for which it is a weak inducer. Cefoxitin too is a labile strong inducer for *Pseudomonas aeruginosa*, *Citrobacter freundii* and *Enterobacter cloacae* but is more stable to the AmpC enzymes of *Serratia marcescens* and *Proteus* species and remains active against these species. The carbapenems imipenem and meropenem are strong inducers but are almost stable to AmpC [241]. They cause enzyme synthesis but are not destroyed and so remain active against ß-lactamase inducible species. Ureidopenicillins, monobactams and second- and third-generation cephalosporins including cefotaxime are labile to AmpC enzymes but are weak inducers [14,241]. They remain active against ß-lactamase-inducible species because they fail to induce ß-lactamase synthesis, not because they are stable to the enzyme. The ß-lactamase inducible species, particularly *Pseudomonas aeruginosa*

and *Enterobacter cloacae*, segregate high frequency spontaneous mutants that manufacture AmpC enzymes continuously at a high level. These mutants are termed variously "constitutive", "stably derepressed" or "enzyme hyper-producers" and are present naturally in inducible populations, usually at frequencies of 10^{-7} but occasionally at up to 10^{-5} [242]. As they produce the ß-lactamase independently of induction they express resistance to the labile weak inducers but not to carbapenems or to stable weak inducers such as temocillin. According to Livermore [241] when a population of inducible bacteria containing, as it invariably does, a few derepressed mutants is challenged with a newer cephalosporin, ureidopenicillin or monobactam, the inducible cells fail to produce enough enzyme to inactivate the drug and are killed whereas the derepressed mutants survive and may overgrow. This process represents selection, not induction. Selection is favoured by labile weak inducers, not as is often mistakenly thought, by strong inducers. Strong inducers, such as imipenem and meropenem, retain equal activity against inducible and derepressed organisms and so do not favour the latter mutants. Likewise weak inducers that are stable, for example temocillin, are not selective [243]. Selections of stably-derepressed mutants have been reported *in vivo* and *in vitro* with most of the third-generation cephalosporins and with the monobactam aztreonam, more rarely with ureidopenicillins, but not with carbapenems nor with the carboxypenicillins [14,15]. The AmpC enzymes are not sensitive to clavulanic acid and are only moderately so to tazobactam [241]. Overall AmpC enzyme derepression was inferred in 65 of the 175 SPEAR isolates.

It is worthwhile repeating that in the present study samples taken on a patient's first day in ITU were taken within four hours of admission to ITU and before SPEAR had been started.

4.7 Individual genera

4.7.1 *Acinetobacter*

It has been said that *Acinetobacter* species have ß-lactamases and are resistant to ß-lactams [244], but it has also been said that the relationship between these bare facts is unclear [170]. In the present study ß-lactamases were detected in 25 of 31 isolates. After the first recognised outbreaks of *Acinetobacter* infections reported in 1971 it was noted that isolates rapidly became resistant to aminopenicillins and the first- and second- generation cephalosporins [244]. Indeed it had been stated that *Acinetobacter* was intrinsically insensitive to most ß-lactams, especially ampicillin and cephalosporins [245]. However early studies reported in 1971 with ampicillin and carbenicillin and later studies with ureidopenicillins such as piperacillin reported in 1980, 1982 and 1985 showed that these compounds were active against 70-80% of *Acinetobacter* strains [244]. At a later stage, when *Acinetobacter* species became resistant to early ß-lactams, the third-generation cephalosporins, particularly cefotaxime [246] and ceftazidime [246,247] were initially active. However within a few years a rapid increase in resistance to carboxypenicillins and to a lesser degree to ureidopenicillins and third-generation cephalosporins, led to the investigation of the possible production of inactivating enzymes by *Acinetobacter* strains [244]. Both TEM-1 and TEM-2 ß-lactamases had been found previously in representative isolates of epidemic strains and Joly-Guillou and colleagues [248] first demonstrated the presence of a novel CARB-type enzyme of pI 6.3 which they designated CARB-5. These three enzymes belong to the group of constitutive plasmid-determined ß-lactamases described by Medeiros [98] and Bauernfeind [249]. In the present study the pI of 5.6 in isolate 191 suggested the presence of a TEM-type ß-lactamase. However this was not in keeping with the large zones of inhibition to carbenicillin and ticarcillin on disk diffusion testing of the isolate.

The rapid increase of resistance to third-generation cephalosporins reported in 1987 led several authors to investigate the possible presence of an inducible cephalosporinase in *Acinetobacter* species [250]. Before the third-generation cephalosporins became available in 1977 an initial study had demonstrated production of an inducible Class I type ß-lactamase in *Acinetobacter* species [251]. The enzyme closely resembled the enzymes produced by *Proteus*, *Citrobacter* and *Pseudomonas* species in terms of molecular mass, sensitivity to inhibitors and inducibility. Medeiros [98] described the "inducible" enzymes of "*Acinetobacter anitratum*" as belonging to the "typical cephalosporinase group" of Sawai and colleagues [252] which included inducible enzymes such as those produced by *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia marcescens* and *Pseudomonas aeruginosa*. However according to Bergogne-Bérézin & Joly-Guillou in 1991 [244] no clear demonstration of an inducible cephalosporinase in *Acinetobacter* species had been reported since that described by Morohoshi and Saito in 1977 [251] and early claims that *Acinetobacter* species had inducible AmpC enzymes were later discounted [253].

According to studies cited by Bergogne-Bérézin and Joly-Guillou [244] successive increases in the resistance of clinical strains of *Acinetobacter* had been reported periodically, high proportions of strains had become resistant to older antibiotics and certain isolates were resistant to most commonly used antibacterial drugs. Less than 50% of strains were inhibited by acceptable concentrations of cefotaxime or even ceftazidime which was initially the most active of the cephalosporins against *Acinetobacter* strains [244]. It was postulated [244] that a particular *Acinetobacter* isolate from a urine specimen might be an ESBL-producer but apparently in general no convincing relationship has been established between antibiogram and ß-lactamase production in *Acinetobacter* species [170].

Isoelectric points of 9.9 and 9.3 have been reported [169] for chromosomal enzymes of *Acinetobacter* species. In the present work ß-lactamases were detected

in 26 of 31 *Acinetobacter* isolates using conventional polyacrylamide gel electrophoresis techniques of isoelectric focusing [156] and broad-range (pH 3.5-9.5) ampholine PAGplates (Pharmacia, Uppsala, Sweden). One ß-lactamase had a pI of 5.6; the others had pI values between 7.7 and 8.9. However the difficulties associated with isoelectric focusing of the chromosomal ß-lactamases of *Acinetobacter* species in conventional systems using broad-range ampholines and the failure to focus enzymes of very high pI values and high molecular masses have been described by Hood and Amyes [253].

Liu and colleagues [240] found in their survey of the prevalence of ß-lactamases among one thousand Gram-negative bacilli that inhibition zone distributions for *Acinetobacter* species were amongst the most complex and variable observed. They found that even those *Acinetobacter calcoaceticus* var *anitratus* isolates that gave substantial zones to ampicillin disks (> 20 mm) commonly gave 4-5 mm larger zones to disks containing amoxycillin/clavulanic acid suggesting ß-lactamase function. However isoelectric focusing resolved clear ß-lactamase bands in only a few of their isolates. An alternative interpretation according to the authors [240] was that these results might "reflect the inherent susceptibility of many isolates of *Acinetobacter* spp. to clavulanate". The ß-lactamase inhibitors sulbactam and tazobactam [254 {p210}, 221] rather than clavulanic acid are known to be inherently active against *Acinetobacter* species. Sulbactam and tazobactam in combination with ticarcillin and as single agents have been found to reduce substantially the MICs of ticarcillin-resistant strains of *Acinetobacter* species [244], tazobactam being more active than sulbactam in both situations. Clavulanic acid (tested in combination with ticarcillin but not as a single agent) was described as causing a significant decrease in the MICs for the ticarcillin-resistant strains but not a true reversion to susceptibility [244]. In the present study all *Acinetobacter* isolates were resistant to ampicillin, 29 of 31 isolates having no zones of inhibition whereas 20 of 31 isolates gave substantial zones (23 mm or greater) to

amoxycillin/clavulanic acid. Isolate 18 which showed no inhibition zones to ampicillin, amoxycillin/clavulanic acid or ticarcillin gave a large zone of inhibition to ticarcillin/clavulanic acid.

The responses of isolates 132 and 137 in the Etest for ESBL production and in disk diffusion testing with clavulanic acid combinations might suggest ESBL function or might indicate that these isolates were particularly sensitive to clavulanic acid. The sensitivity of both isolates to carbenicillin, ticarcillin and piperacillin and resistance to cefoxitin was not in keeping with ESBL production. Neither resistance to cefotaxime nor to ceftazidime was transferred by transconjugation from these two *Acinetobacter* isolates. Both isolates had been grown from rectal swabs taken from a 29-year-old patient (patient 81) on her sixth and eighth days in ITU with respiratory failure. Hodgkin's disease had been diagnosed four years earlier and she had undergone a bone marrow transplant six weeks prior to her ITU admission. Ceftazidime had been started six days before she was admitted to the ITU and was continued along with SDD throughout her ITU stay. The patient had also received parenteral tobramycin. Interestingly this was the same patient (patient 81) from whom the two isolates (133 and 140) of *Klebsiella pneumoniae* which were positive in the Etest for ESBL production and whose resistance to ceftazidime was transferable had been grown from samples taken on her sixth and eleventh days in ITU. However isolate 132 had an enzyme of pI 7.9 and isolate 137 had enzymes of pIs 7.9 and 8.6 whereas the transferable ß-lactamases from the *Klebsiella* isolates 133 and 140 had a pI of *c.*5.3.

Although chromosomal ß-lactamase induction in general has been more or less discounted in *Acinetobacter* species and ESBL production was not demonstrated in this study, it could be argued that the widespread use of cefotaxime created a selection pressure for *Acinetobacter* strains resistant to cefotaxime. However 16 of the 31 *Acinetobacter* isolates were obtained from samples taken from 16 patients on their first day in ITU and were unlikely to be related to SPEAR. From plasmid and

ß-lactamase profiles and antibiograms there was no evidence of an epidemic strain among the 15 isolates obtained from samples taken on subsequent days from the other seven patients.

4.7.2 *Citrobacter freundii*

Matthew and Harris [157] reported pIs of *c.*7.5 and 8.3 for chromosomally-mediated ß-lactamases of two strains of *Citrobacter freundii* and a pI of 8.6 has been described for a chromosomally-mediated ß-lactamase of *Citrobacter freundii* GN7391 [255]. Isoelectric points of 8.9 and 8.6 have also been recorded for chromosomal enzymes of two other strains [169].

In the present study the results for nine of the 10 isolates of *Citrobacter freundii* suggested chromosomal AmpC ß-lactamase derepression possibly directly related to SPEAR in four of the isolates. Regarding the other five isolates (isolates 4 and 5 from patient 56 and 118, 119 and 122 from patient 87) the resistant isolates were first grown from samples taken on the respective patients' first days in ITU and were therefore unlikely to be related to SPEAR. The resistance of isolate 9 to piperacillin and the third-generation cephalosporins, the high pI (8.3) of the enzyme and the "not determinable" result in the Etest for ESBL production allowed for the interpretation of chromosomal AmpC ß-lactamase derepression, but the isolate's sensitivity to cefoxitin, carbenicillin, ticarcillin, ticarcillin/clavulanic acid, piperacillin/tazobactam and aztreonam was at variance with this theory.

4.7.3 *Enterobacter* species

In the present study the pIs of the ß-lactamases obtained from the *Enterobacter* isolates ranged from 4.9 to 8.8. Matthew and Harris [157] reported a pI range between 7.4 and 7.6 for chromosomally-mediated ß-lactamases of *Enterobacter cloacae*. Different types of chromosomal ß-lactamases have been described in *Enterobacter* species. They have been designated type A, pI 8.8 and type B, pI 7.8 [256] and type B pI 7.9 [257]. Other pI values of 8.25 [258], 8.2 [259], 8.4 [260]

and 8.3 [261] have been published reflecting differences of methodology according to Then and colleagues [257]. Other enzymes with pIs of 6.9 and > 9.0 have been recorded [259]. *Enterobacter* species have inducible ß-lactamases and strains of *Enterobacter* species with derepressed chromosomal ß-lactamases show reduced sensitivity to all ß-lactams except carbapenems [170], mecillinam [262,263] and temocillin [243]. Usually the ß-lactamase inhibitors clavulanic acid, sulbactam and tazobactam do not have sufficient inhibitory activity to potentiate their partner penicillins against derepressed organisms.

Enterobacter species may also harbour plasmid-mediated ß-lactamases. The widespread TEM-1, TEM-2 and SHV-1 plasmid-mediated enzymes attack narrow-spectrum cephalosporins, cefoperazone and cefamandole and all the anti-Gram-negative penicillins except temocillin. Aminothiazolyl cephalosporins (cefotaxime, ceftazidime, ceftriaxone), cephamycins, monobactams and carbapenems are considerably more stable. However much more worryingly they may harbour ESBLs.

Isoelectric points for TEM enzymes including the parental enzymes TEM-1 and TEM-2 and the TEM-derived ESBLs, range from 5.2-6.5 [169] and pIs for the SHV enzymes including the parental SHV-1 enzyme and the SHV-derived ESBLs range from 7.0-8.2 [169]. The TEM- and SHV-derived ESBLs all have the ability to attack most of the ß-lactams including the extended-spectrum cephalosporins and aztreonam. The carbapenems and cephamycins such as cefoxitin are stable and the enzymes are inhibited by clavulanic acid.

Therefore an additional difficulty in the identification of *Enterobacter* ß-lactamases in the present study, especially as most of the isolates contained plasmids, was the possibility of the coexistence of plasmid-mediated enzymes with a derepressed AmpC enzyme. A TEM-1, TEM-2 or SHV-1 enzyme would not confer resistance to cefoxitin or to the extended-spectrum cephalosporins or to aztreonam; an ESBL on its own would not confer resistance to cefoxitin and would be likely to

be detected in the Etest for ESBL production, but the effect on the antibiogram of either or both of these would be masked by that of a derepressed chromosomal AmpC enzyme. The production of ESBLs in *Enterobacter* species has been identified and is capable of producing clinically relevant resistance [23,190,264,265].

Cefoxitin sensitivity and inhibition by clavulanic acid are properties used to help identify ESBLs. However if an ESBL were produced by an isolate which also had chromosomal ß-lactamase derepression neither cefoxitin testing nor the Etest for ESBL production would be discriminatory. Although ESBLs are found most commonly in klebsiellae and next in *Escherichia coli* they occur also in other enterobacteria including *Enterobacter* species [23,190,264-266]. Kitzis and colleagues [264] ascribed the high cefoxitin MICs of their ESBL-producing *Enterobacter*, *Citrobacter* and *Serratia* species to cephalosporinase induction, not derepression. De Champs and colleagues [23] commented that the frequency of ESBL production in *Enterobacter aerogenes* strains in their study was probably underestimated because detection of the synergy between cephalosporins and clavulanic acid might have been hidden by production of the cephalosporinase. D'Agata and colleagues [266] reported a range of cefoxitin MICs from equal to or less than 2 mg/L to equal to or greater than 32 mg/L for their ESBL-producing strains which included *Enterobacter* and *Citrobacter* species. The authors commented that the differences in cefoxitin MICs probably reflected porin changes associated with resistance, as described by Pangon and colleagues [267] in an ESBL-producing strain of *Klebsiella pneumoniae*.

Isolates 43 and 47 had multiple ß-lactamase bands with pIs ranging from 5.6-8.8. The isolates each had one similar band of pI 8.8 which most likely denoted a chromosomally-mediated enzyme but the pIs of the other bands were different in each isolate. The isolates had different plasmid profiles and were not obviously epidemiologically related. The fact that cefotaxime resistance was not transferred

and the antimicrobial susceptibility pattern suggested chromosomal ß-lactamase derepression as a cause of cefotaxime resistance. However with such a range of ß-lactamases similar arguments pertain here as above regarding the possible presence of TEM or SHV enzymes.

Another five isolates of *Enterobacter* species (77, 79, 81, 116 and 121) had an unusual but similar antibiogram. Their ceftazidime MICs were 2, 2, 3, 3 and 4 mg/L respectively and they were sensitive to aztreonam and piperacillin, but resistant to cefoxitin, cefotaxime and gentamicin. The reason for this pattern of resistance was not clear. Possibly it reflected changes in outer membrane permeability [261,268] along with chromosomal ß-lactamase derepression and/or ESBL production. Isolates 77, 79 and 81 were from the same patient (patient 62) as isolates 78 and 80 which were much more resistant to ceftazidime (MICs > 32 mg/L) and resistant to aztreonam and piperacillin as well as cefoxitin, cefotaxime and gentamicin. ESBL production was not determinable by Etest in any of the isolates. All had large plasmids but cefotaxime resistance was not transferred. Isolates 77-81 had a ß-lactamase of pI 7.6 and isolates 78-81 also had a ß-lactamase of pI 8.0. Isolates 116 and 121 from two other patients (patients 61 and 67) had ß-lactamase bands of pI 7.9. There was no obvious clinical or epidemiological connection between patient 62 from whom isolates 77-81 were grown and the two other patients. Isolates 77-81 were obtained from samples taken from patient 62 on her first and second days in ITU. Isolates 77 and 78 showing both patterns of resistance (isolate 77 being more sensitive to ceftazidime and isolate 78 being much more resistant) were both isolated on the patient's first day in ITU, which would argue against the implication of SPEAR in their resistance (antibiotic therapy prior to admission to the ITU was not recorded), whereas isolates 116 and 121 were obtained from samples taken from patients 61 and 67 on their fourth and fifth days respectively in the ITU. There were some similarities also between these isolates and a strain of *Enterobacter aerogenes* from a patient described by Mellencamp and colleagues [269]. This strain had an

unusual susceptibility profile in that it was resistant to ceftazidime but susceptible to other third-generation cephalosporins and piperacillin in routine disk diffusion tests. During therapy with piperacillin and amikacin the strain developed resistance to multiple ß-lactam antibiotics in association with the loss of an outer membrane protein. According to Sanders [270] hyperproduction of AmpC ß-lactamase in this strain was not sufficient to confer resistance to multiple ß-lactam agents until coupled with a decrease in permeability. In the present study the resistance to cefotaxime along with relative sensitivity to ceftazidime in isolates 77, 79, 81, 116 and 121 would seem more difficult to explain by limited chromosomal AmpC ß-lactamase hyperproduction. As the diffusion rate of ceftazidime through the outer membrane into the periplasm is slower than that of cefotaxime [92] a greater relative increase in ceftazidime rather than cefotaxime resistance might be expected. However the diffusion studies of Yoshimura and Nikaido [92] were performed in *Escherichia coli* K-12 and may not pertain to clinical isolates of *Enterobacter* species. In 1979 Preheim and colleagues [271] reported the emergence of a type of resistance in two patients during therapy with moxalactam that also conferred cross-resistance to the aminoglycosides. In 1985 Sanders and Sanders [268] commented that this type of cross-resistance between ß-lactams and aminoglycosides was important but distinct from that associated with ß-lactamase induction and derepression, was not associated with drug-inactivating enzymes and appeared to be caused by changes in outer membrane permeability. The latter explanation might be applicable to isolates 134, 135, 141 and 142 which were resistant to gentamicin and all the ß-lactams (including ceftazidime) other than the carbapenems and mecillinam. Their resistance may however be the result of a combination of chromosomal AmpC ß-lactamase derepression and outer membrane permeability changes.

Chow and colleagues [17] observed that in 40 case reports from various authors, the duration of third-generation cephalosporin therapy before *Enterobacter* species

resistant due to increased ß-lactamase production emerged at clinically infected sites, ranged from 1 to 20 days. In the present study, samples taken on the patient's first day in ITU were taken within four hours of the patient's admission to ITU and before SPEAR had been started.

Multiresistance which includes resistance to extended-spectrum cephalosporins and aminoglycosides also raises suspicion of the presence of ESBLs [265] in those isolates with ß-lactamases of appropriate pI values. As reported by Philippon and colleagues [265] the TEM- and SHV- derived ESBLs are encoded by multiresistance plasmids which are usually readily transmissible and which may carry resistance to aminoglycosides. Outbreaks of infection caused by ESBL-mediated resistant *Enterobacter* species have been noted predominantly in France. Of the three most common ESBLs, CTX-1 (TEM-3), CAZ-5 (SHV-4) and CAZ-6 (TEM-24), detected in the *Enterobacteriaceae* between 1988 and 1989 in one French hospital, 20% of the CTX-1 and 83% of the CAZ-6-producing isolates were *Enterobacter* species [23,190,264,265]. These enzymes effectively hydrolyzed all ß-lactams with the exception of the cephamycins and imipenem. Therefore acquisition of plasmid-encoded enzymes by *Enterobacter* species is possible [18].

In the present study an argument against ESBL production in these *Enterobacter* isolates was the fact that cefotaxime resistance was not transferred from any of the *Enterobacter* isolates to the recipient *Escherichia coli*. However in a study of TEM-10, TEM-12 and TEM-26, only 25 of 80 isolates transferred these ß-lactamase genes by conjugation [239]. Also Piddock and colleagues [272] in their UK survey of the occurrence of ESBLs observed that it was exceptionally difficult to transfer the gene encoding cephalosporin resistance to a new host and suggested that these genes moved on to replicons which were incapable of easy conjugal transfer, but could continue to move rapidly among bacterial species and strains leading to very complicated epidemiological situations [272].

In summary twenty-three *Enterobacter* isolates with resistance to cefotaxime possibly directly related to SPEAR were isolated from twelve patients. Chromosomal AmpC derepression was likely in twenty-one isolates (39, 40, 43, 50, 51, 61, 62, 64, 65, 66, 92, 94, 126, 134, 135, 141, 142, 149, 150, 161 and 199) from seven patients. There was the possibility of coexisting plasmid-mediated ß-lactamase production in four isolates (43, 92, 94, and 126) from another three patients because of the pI values and plasmid profiles, although cefotaxime resistance was not transferred. It was difficult to explain satisfactorily the antibiogram of isolates 116 and 121 from patients 61 and 67.

It is unlikely that the reduced susceptibility to cefotaxime of the other twenty-eight *Enterobacter* isolates obtained from another thirteen patients was directly related to SPEAR. Fourteen of these isolates were grown from samples taken on the first day of admission to the ITU and the others from samples taken on subsequent days. In eight cases where more than one isolate was obtained from the same patient the isolates appeared similar according to plasmid and ß-lactamase profiles and antibiograms. The inference from the available data was that chromosomal AmpC derepression was the most likely cause of the reduced susceptibility to cefotaxime except for isolates 77, 79, 81 (patient 62), 47 (patient 75), 116 (patient 61) and 121 (patient 67).

4.7.4 *Escherichia coli*

Isolate 76 had a cefotaxime MIC of 4 mg/L but was sensitive to ceftazidime and cefoxitin. Cefotaxime resistance was not transferred and the isolate was negative in the Etest for ESBL production. Other than temocillin it was resistant to all the penicillins and the inhibitor combinations, amoxycillin/clavulanic acid, ticarcillin/clavulanic acid and piperacillin/tazobactam. It had ß-lactamases of pIs 5.4 and 7.4 the latter aligning with OXA-1 on the isoelectric-focusing gel. *Escherichia coli* isolates usually have only insignificant levels of uninducible AmpC enzymes

[172,173] and resistance to ß-lactams, unless due to impermeability or failure to bind to PBPs, is most often caused by acquisition of secondary ß-lactamases [240,273]. It is possible that isolate 76 had an inhibitor-resistant TEM mutant enzyme along with an OXA-type enzyme. The classical TEM-1 and TEM-2 ß-lactamases segregate mutants which are resistant to ß-lactamase inhibitors and ten variants have been described [274-277]. Resistance to inhibitor combinations is often caused by high-level production of classical TEM-1 enzymes themselves [278,279] and such resistance is commoner with clavulanic acid than with tazobactam combinations. Neither the classical TEM nor the inhibitor-resistant mutant TEM enzymes should compromise cefotaxime sensitivity. The OXA-1 enzyme has been said to hydrolyze cefotaxime at a significant rate [280] although in his review in 1995 Livermore [170] stated that extended-spectrum cephalosporins were spared by OXA-1 ß-lactamase. The OXA- enzymes 4-7 have been reported to be active against cefotaxime [98]. By disk diffusion testing isolate 76 was totally resistant to mecillinam which binds exclusively to PBP-2.

Isoelectric points for chromosomally-mediated ß-lactamases of *Escherichia coli* have been recorded as 8.6 [157], 8.3 and 8.1 [155], 9.2 [167], 8.5, 8.7 and 8.8 [169]. Isolate 200 more closely resembled those occasional isolates of *Escherichia coli* which do produce copious amounts of the chromosomally-mediated AmpC enzyme and are more resistant to antibiotics [172]. Such isolates show reduced susceptibility to all ß-lactams except carbapenems, temocillin and mecillinam [170]. Isolate 200 was resistant also to temocillin. According to Livermore [170] AmpC hyperproducers account for less than 2% of all *Escherichia coli* isolates in most surveys. Such mutant isolates of *Escherichia coli* resemble derepressed *Enterobacter* species in phenotype but not in genetic organization and their ß-lactam MICs are usually lower than those of ß-lactamase derepressed *Enterobacter* species. The reason for their rarity is the fact that AmpC hyperproduction in *Escherichia coli* requires two separate mutations [281] and the selection of such mutants during

therapy has not been described [241]. One ß-lactamase band of pI 8.5 was detected in isolate 200, cefotaxime resistance was not transferred and the isolate was negative in the Etest for ESBL production.

4.7.5 *Flavobacterium odoratum*

Flavobacterium species are found in the hospital environment and are commonly resistant to many antimicrobial agents including cefotaxime, aztreonam, the carbapenems and gentamicin. It was therefore no surprise that isolates 89 and 90 were resistant to most of the antibiotics tested. A chromosomal class B (zinc) ß-lactamase of pI 5.8, active against carbapenems as well as penicillins and cephalosporins and resistant to clavulanic acid, has been reported in *Flavobacterium odoratum* [282]. In this study a ß-lactamase band of pI 7.8 of unknown identity was detected in isolate 89, but not in isolate 90 from the same patient (patient 73). Plasmids were not detected in either isolate, cefotaxime resistance was not transferred and ESBL production was not determinable by Etest.

4.7.6 *Hafnia alvei*

A pI of 7.1 has been published for the chromosomal ß-lactamase of a strain of *Hafnia alvei* 1094E [155,157] but the genus *Hafnia* is not mentioned in some more recent classifications and reviews [167,169,170]. Piddock and colleagues [272] described an enzyme of pI 7.9 in three of seven strains of *Hafnia alvei* resistant to cefotaxime and ceftazidime and inferred that this enzyme might be chromosomal in view of negative polymerase chain reaction results with SHV primers. Two of these strains also had enzymes with pIs > 8 [272].

As cefotaxime resistance was not transferred in the present study and ESBL production was not determinable by Etest in the four *Hafnia* isolates it is possible that the enzyme of pI 7.8 in all four isolates was chromosomal and that hyperproduction caused resistance to the third-generation cephalosporins. The cefoxitin sensitivity of the isolates is an argument against this theory, although

cefoxitin has been reported to be almost stable to the ß-lactamases of some inducible species, namely *Serratia*, *Providencia* and *Morganella* [170]. Isolate 75 from patient 86 had another enzyme of pI 7.4 which was possibly an SHV-type or OXA-type ß-lactamase. The patient's therapy prior to ITU admission was not recorded but the isolate was obtained from a sample taken on his first day in ITU and was therefore unlikely to be related to SPEAR. Isolates 167, 168 and 169 were obtained from a different patient (patient 43) on her first and second days in ITU and were therefore also unlikely to be related to SPEAR. This patient had received only a first-generation cephalosporin prior to her ITU admission.

4.7.7 *Klebsiella pneumoniae* and ESBLs

The cefotaxime MICs for isolates 133 and 140 were 0.06 mg/L and 0.25 mg/L respectively but their ceftazidime MICs were 8 mg/L and > 32 mg/L respectively. Both isolates were positive in the Etest for ESBL production. Ceftazidime resistance was transferable from both isolates to the recipient organism *Escherichia coli* J62-2 and associated with this resistance a ß-lactamase of calculated pI 5.2 (although it aligned with the control TEM-1 ß-lactamase of known pI 5.4) was detected in the transconjugants from both isolates in addition to the ß-lactamase of pI 8.4 which was already present in the recipient organism. Transfer of a large plasmid, perhaps two large plasmids, was demonstrated in the transconjugants from isolate 140, but plasmids were not detected in transconjugants from isolate 133. The genetics may be complex [239] but it is very likely that isolates 133 and 140 harboured one or more TEM-derived ESBLs. Although synergy was demonstrated between disks of amoxycillin/clavulanic acid and ceftazidime, the donor isolates 133 and 140 showed reduced sensitivity to amoxycillin/clavulanic acid by disk diffusion testing. This might seem unusual as TEM- and SHV-derived ß-lactamases are said to be inhibited well by clavulanic acid [169]. In their review Philippon and colleagues [265] stated that ESBLs remained susceptible to ß-lactamase inhibitors, although they pointed

out that the effect of the inhibitor might be reduced in a strain which made TEM-1 as well as an ESBL [264]. However since then it has been reported that MICs of clavulanic acid combinations for ESBL-producers are often high [190,283]. The transconjugants obtained from isolates 133 and 140 in the present study were both borderline sensitive to amoxycillin/clavulanic acid (i.e. more sensitive than the donor strains) by disk diffusion testing.

Most ESBLs are mutants of TEM-1, TEM-2 and SHV-1 enzymes and over 25 different variants have been claimed and are numbered TEM-3 to TEM-27 and SHV-2 to SHV-7. The earliest documented ESBL-producing isolate in the world is now recognized to be that recovered in Liverpool in 1982 [284]. Extended-spectrum ß-lactamases have been responsible for a number of incidents where bacteria resistant to third-generation cephalosporins have caused therapeutic problems in hospitals around the world [170,190,285].

It is probable that the widespread use of third-generation cephalosporins has provided selective pressure promoting emergence of this type of resistance [265]. Payne and Amyes [285] suggested that the degree of control in the use of later generation cephalosporins would probably determine whether ESBLs remained a minor inconvenience or emerged as a major threat. However there are studies in which prior exposure to third-generation cephalosporins was found not to be a risk factor for colonization by ESBL-producers [266,286]. There is also evidence that carriage of AGNB that express resistance to third-generation cephalosporins is far more common than infection caused by such bacteria, suggesting that the evolution of resistance to third-generation cephalosporins may be largely cryptic [239]. Patient 81 in the present study from whom isolates 133 and 140 were grown was an oncology patient who had been treated with ceftazidime and tobramycin for a number of days prior to her ITU admission. This therapy, along with the SDD component of SPEAR, was continued during her stay in ITU. Tobramycin was also a component of SDD. The organisms were isolated from samples taken on days six

and eleven in ITU. It is possible that the ceftazidime and tobramycin instituted in the oncology ward, along with the general selection pressures prevalent in the ITU as well as the specific selection pressures of SDD all contributed to her ESBL carriage.

Typically ESBLs are recognised by the unusual antibiotic resistance they provide in their hosts, conferring the ability to attack extended-spectrum cephalosporins and monobactams as well as narrow-spectrum cephalosporins and anti-Gram-negative penicillins, but in some isolates an intermediate or even susceptible phenotype can result [265]. The enzymes vary considerably in the level of resistance conferred to cefotaxime, ceftazidime and aztreonam. Some enzymes do not increase the cefotaxime MIC at all with a conventional bacterial inoculum [190]. Recognizing that a classification scheme for ESBLs based entirely on structure was unhelpful, Payne and Amyes [285] proposed a categorization scheme by which TEM- and SHV- derived ESBLs were assigned to Groups 1 to 4 according to their relative efficiencies of hydrolysis of cefotaxime and ceftazidime. In 1995 Du Bois and colleagues [287] proposed an additional group (Group 0) to accommodate enzymes with activities almost indistinguishable from TEM-1 or TEM-2. One of the most important problems in the detection of ESBLs in clinical strains is the possibility of a low expression of the enzyme [288] which may occur even in different isolates of the same epidemic strain and may not be differentiated easily from susceptible strains by disk diffusion testing [288]. In this study isolate 133 appeared sensitive to ceftazidime by disk diffusion testing. As described by D'Agata and colleagues [266] not all ESBL producers appear resistant to all cephalosporin and penicillin antibiotics even when MICs are performed. Yet while ESBL-producers may fail to reach the pharmacological breakpoints for resistance favoured by the NCCLS [194] they may have biological resistance that will cause treatment failure with significantly increased mortality [287,289,290].

4.7.8 *Morganella morganii*

It was impossible to predict enzyme identity in this ß-lactamase inducible species from the antibiograms and range of pI values obtained. Matthew and Harris [157] reported a pI value of 7.3 for a chromosomal ß-lactamase of a strain of *Morganella morganii* and pIs of 8.7 and 7.2 have been recorded for the chromosomal enzymes of two other strains [169]. According to work reviewed by Livermore [170] the levels of AmpC ß-lactamase in *Morganella* strains are in general about tenfold below those in derepressed *Enterobacter* species and *Citrobacter freundii*. Apparently cefoxitin is almost stable to the *Morganella* enzyme and is moderately active against both ß-lactamase-inducible and -derepressed strains, whereas it is inactive against both inducible and derepressed strains of *Enterobacter* species and *Citrobacter freundii*. All isolates other than 23, 57 and 58 in this study were sensitive to cefoxitin by disk diffusion testing. Isolates 23 and 57 showed no zones of inhibition to cefoxitin and isolate 58 gave only a small zone. According to Akova and colleagues [291] tazobactam inhibits the *Morganella morganii* AmpC ß-lactamase sufficiently to potentiate piperacillin against derepressed strains whereas it has little effect against the AmpC enzymes of *Enterobacter* species and *Citrobacter freundii*. In the present study all isolates other than 23, 57 and 58 were fully sensitive to piperacillin/tazobactam by disk diffusion testing. Isolates 10, 58, 113 and 157 gave large zones of inhibition with carbenicillin by disk diffusion testing although isolates 10 and 113 were resistant to piperacillin and isolate 157 showed reduced susceptibility. Cefotaxime resistance was not transferred and ESBL production was not determinable by Etest in all isolates. It was tempting to infer that resistance to cefotaxime and ceftazidime was caused by AmpC derepression but difficult to explain the apparent carbenicillin sensitivity of five isolates.

Cefotaxime resistance was possibly directly related to SPEAR in four isolates of *Morganella morganii*, 57 and 58 (patient 42), 113 (patient 68) and 157 (patient 21)

but unlikely to be so in isolate 16 which was obtained from patient 33 on his first day in the ITU. It was difficult to know what was happening with isolates 23, 24 and 25 grown from samples taken from patient 82 on her first, third and first day of readmission to the ITU respectively. Isolates 23 and 24 had identical plasmid profiles while isolate 25 differed slightly but had been examined on a different gel. Isolates 23 and 25 each had two enzymes of pIs 6.5 and 7.8 and isolate 24 had one enzyme of pI 7.8. In general isolate 23 was much more resistant than isolates 24 and 25.

4.7.9 *Pseudomonas aeruginosa*

It was no surprise that many isolates of *Pseudomonas aeruginosa* in the present study showed reduced sensitivity or resistance to cefotaxime which is not used clinically as an anti-pseudomonal agent. According to reports cited by Kucers and colleagues [254 {p321}] most strains are moderately resistant (MICs 16-32 mg/L). Some studies have found approximately 20% to be highly resistant (MICs 64 mg/L or higher) while others have noted the majority of strains to be highly resistant. Some strains may have intrinsic resistance to cefotaxime in the form of low outer membrane permeability [292]. Most strains are susceptible to ureido- and carboxypenicillins, ceftazidime, aztreonam, imipenem and meropenem. Increased resistance to cefotaxime and resistance to the other agents can arise by various mechanisms, including mutational derepression of the AmpC chromosomal ß-lactamase, acquisition of secondary plasmid- or transposon-mediated ß-lactamases, reduced permeability, multi-drug efflux or, in the case of imipenem, loss of the D2 porin [293].

Pseudomonas aeruginosa has an inducible AmpC enzyme similar to that of *Enterobacter* species. This chromosomal AmpC enzyme is universally present in *Pseudomonas aeruginosa* and is normally, without induction, expressed weakly [293]. As with *Enterobacter* species ampicillin and narrow-spectrum cephalosporins

are labile to hydrolysis and induce the enzyme strongly, destroying their own activity, whereas ureidopenicillins and extended-spectrum cephalosporins are labile but induce weakly and so are active against inducible strains but not against derepressed mutants [170]. Carbapenems are strong inducers that are marginally labile (imipenem) or are effectively stable (meropenem) [170]. Derepression in *Pseudomonas aeruginosa* is often only partial, such that the uninduced enzyme level is higher than is normal for the species but substantial inducibility is retained [273,294] whereas derepression in *Enterobacter* species is almost always total [170]. Even a small degree of derepression compromises ureidopenicillins, whereas only total derepression noticeably compromises carbenicillin [294]. Selection of totally or partially derepressed mutants can occur during anti-pseudomonal therapy with labile weak inducers. Extended-spectrum cephalosporins, ureidopenicillins (mezlocillin and azlocillin) and piperacillin, have been widely reported to select for these mutants in *Pseudomonas aeruginosa* infections [14,295].

Isoelectric points for the chromosomally determined AmpC (Sabath and Abraham's) ß-lactamase of *Pseudomonas aeruginosa* have been reported to range from 7.4 to 7.9 [296], 7.2 to 8.15 [157] and 7.4-8.8 [294,297].

Plasmid-mediated and other secondary ß-lactamases (i.e. enzymes encoded by chromosomal inserts) have been reported widely in *Pseudomonas aeruginosa* but are much rarer than in enterobacteria according to Livermore in his review [170]. Secondary ß-lactamases were found in multicentre surveys in the United Kingdom in 2.5% of 1,866 *Pseudomonas aeruginosa* isolates collected in 1982 [294] and 0.7% of 1,991 isolates collected in 1993 [293]. It has been suggested that the limited transmissibility of many plasmids into and within *Pseudomonas aeruginosa* has acted to restrict the enzyme dissemination [294]. Plasmid-mediated PSE-1, PSE-2, PSE-3 and PSE-4 enzymes are well known [175,294,298] and numerous OXA types have been recorded [293,294,298] as have various obscure types such as NPS-1 and LCR-1 [170]. PSE-1 and PSE-4 enzymes are said to predominate mainly because of

clonal selection of producers rather than because of plasmid spread [170]. TEM and SHV types do occur but are rare compared with their predominance in enterobacteria [170].

In the present study plasmids were detected in only two of forty-nine isolates of *Pseudomonas aeruginosa* and in none of the other eight *Pseudomonas* species. No enzymes with pIs suggestive of PSE-1, 2, 3 or 4 were detected. Isolates 163 and 183 had enzymes of 7.4 as well as 7.9. However the enzymes of pI 7.4 were unlikely to be OXA-type enzymes as the isolates showed good zones of inhibition to ticarcillin, azlocillin and piperacillin. There was no apparent epidemiological or temporal connection among the 22 isolates which showed reduced sensitivity to carbenicillin and or ticarcillin. In eleven out of the fifteen patients involved the organism had been isolated from a sample taken on the first day of admission to the ITU; the patients had come from a variety of wards and sometimes different hospitals. From the other four patients such isolates had been grown from samples taken on days 10, 2, 5 and 3 of their ITU admissions. None of the fifteen patients' stays overlapped. Carbenicillin is not inactivated by the chromosomally mediated AmpC ß-lactamase of *Pseudomonas aeruginosa* isolates. Resistance to carbenicillin in *Pseudomonas aeruginosa* may be plasmid-mediated or intrinsic [254 {p145}]. Intrinsic resistance may result from an alteration of cell wall permeability or a marked decrease in the affinity of PBPs. In some strains highly resistant to carbenicillin both of these mechanisms of intrinsic resistance may be involved together with the production of plasmid-mediated ß-lactamases. Ticarcillin is consistently at least twice and sometimes four times as active as carbenicillin against *Pseudomonas aeruginosa* but strains of all bacteria including *Pseudomonas aeruginosa* that have become resistant to carbenicillin are also ticarcillin-resistant [254 {p146}].

Twelve isolates of *Pseudomonas aeruginosa* (17, 19, 20, 27, 42, 48, 49, 103, 117, 151, 171, 182) were resistant to ceftazidime (MIC >32 mg/L). All were non-determinable in the Etest for ESBL production. In contrast to cefotaxime, a high

degree of activity against *Pseudomonas aeruginosa* is one of the most important properties of ceftazidime and the main mechanism of resistance seems to be an increased production of chromosomally mediated AmpC ß-lactamase [254 {p322}]. Three of the ceftazidime-resistant organisms in this study (isolates 27, 49 and 117) were resistant also to azlocillin, piperacillin and aztreonam and two (isolates 48 and 103) were resistant to azlocillin and aztreonam. Four had ß-lactamases of pI equal to or greater than 7.9 and in one (isolate 49 from patient 28) ß-lactamases were not detected. Isolate 27 was grown from a sample taken from patient 58 on her eighth ITU day and isolate 117 from a sample taken from patient 61 on his fifteenth ITU day. Isolates 48 and 49 were obtained from samples taken from patient 28 on his eleventh and sixteenth days in ITU. Isolate 103 was grown from a sample taken from patient 51 on her seventh day in ITU and its antibiogram was quite different from that of the much more sensitive isolate 98 which was grown from the same patient on her first day in ITU. It was likely that the resistance pattern of the five isolates (27, 117, 48, 49, 103) was caused by chromosomal AmpC derepression. One of these isolates (isolate 48) showed reduced sensitivity and three (isolates 27, 49 and 117) were resistant to carbenicillin by disk diffusion testing. Carbenicillin is reported to be stable to and a potent inactivator of Sabath and Abraham's ß-lactamase [299] but it can be compromised by total derepression of the enzyme [294]. It is possible also that porin deficiency could have contributed to the resistance in these isolates. As the other seven ceftazidime-resistant isolates gave large zones of inhibition to aztreonam and to piperacillin which is compromised by even a small degree of AmpC derepression [294], it is unlikely that their resistance was caused by AmpC derepression. Another organism (isolate 124) showed reduced sensitivity to ceftazidime (MIC 12 mg/L) and resistance to aztreonam by disk diffusion but otherwise was a fairly sensitive strain. It is noteworthy that seven of the 12 ceftazidime-resistant (MIC >32 mg/L) isolates were sensitive by disk diffusion testing: five of the seven had inhibition zones of 30 mm diameter i.e. 12

mm greater than the minimum interpreted as sensitive (18 mm) by NCCLS criteria (Table 2). Occasional misleading results of disk susceptibility tests have been found in other studies with other third generation cephalosporins: moxalactam [300] and cefoperazone [301].

Isolate 56 was resistant to imipenem and to meropenem. In a study of resistance mechanisms in *Pseudomonas aeruginosa* reported in 1995 [293] it was found that resistance to imipenem was largely dissociated from that to other ß-lactam agents and probably reflected loss of D2 porin, whereas resistance to meropenem was mostly associated with intrinsic resistance to penicillins and cephalosporins.

Thirty-seven of the isolates were negative in the Etest for ESBL production but twelve were not determinable. Five of the latter (isolates 19, 20, 27, 117 and 182) had ß-lactamases with pIs between 7.6 and 8.2 and theoretically might have produced an SHV-type ESBL although cefotaxime resistance was not transferred from any of the *Pseudomonas* isolates and two small plasmids (*c.*7 kb and 40 kb) were detected in isolate 117 only. The production of ESBLs would have been unlikely in isolates 19, 20 and 182 as they gave large zones of inhibition with azlocillin, piperacillin and aztreonam disks. In keeping with chromosomal AmpC derepression, resistance to ticarcillin and piperacillin was not inhibited even to a small extent by clavulanic acid or tazobactam in isolates 27 and 117.

As twenty-three of the forty-nine *Pseudomonas* isolates were grown from samples taken from patients on their first day in ITU, resistance to cefotaxime was unlikely to be related to SPEAR. At worst the general selection pressures created by SPEAR may have contributed indirectly to the isolation of the other twenty-six pseudomonads, only five of which probably had derepression of their chromosomal AmpC ß-lactamases directly related to the use of cefotaxime in SPEAR.

4.7.10 *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia has become a focus of interest because of the increasing frequency of its isolation and its broad-spectrum antimicrobial resistance. Severe infections may be caused by this organism in seriously ill or immunocompromised patients. Susceptibility tests for these organisms vary enormously with the test medium and method used [302,303]. All isolates in the present study were tested at the same time, using the same methods and the same batches of media. Few antimicrobial agents are effective against this opportunistic pathogen which is notorious for its resistance to ß-lactam antibiotics, including carbapenems. It has been suggested that low outer-membrane permeability and production of two chromosomally-mediated inducible ß-lactamases, L-1 and L-2, contribute to this resistance [304]. L-1 is a class B zinc enzyme, broadly active against penicillins, carbapenems and many cephalosporins but not against aztreonam. L-2 is an unusual cephalosporinase which can hydrolyze those cephalosporins and monobactams that escape L-1 [305]. Cefotaxime is a potent inducer of these ß-lactamases [305] and because of the high level of hydrolytic activity of L-1 for cefotaxime [306], this compound is inactivated quickly by induced enzyme and overproduction of enzyme does not further increase the high resistance of inducible strains. Therefore cefotaxime does not exert a selection pressure for such strains. This contrasts with the behaviour seen with the inducible chromosomal AmpC ß-lactamases typical of *Pseudomonas aeruginosa* and some enterobacteria [14]. The pIs of the chromosomally-mediated ß-lactamases L-1 and L-2 have been recorded respectively as 6.9 and 9.2 [168], 6.5 and 8.5 [304] and 6.9 and 8.4 [169].

Isolates 28-32 in the present study were from the same patient (patient 36) and a ß-lactamase of pI 6.1 was detected in all five isolates on two different isoelectric focusing gels. What did this ß-lactamase band of pI 6.1 represent? Isolates 28 and 29

were grown from samples taken on the patient's first day of admission to ITU (and therefore unlikely to be related to SPEAR), isolate 30 on the third day and isolates 31 and 32 on her sixth and eighth days respectively. Isolate 45 was grown three months later from a sample taken from another patient (patient 95) on his second day in ITU and isolate 99 was grown seven months after that from yet another patient (patient 51) on her second day in ITU but not on subsequent days. ß-Lactamase bands were not detected in isolates 45 and 99. Plasmids were not detected in any of the isolates, cefotaxime resistance was not transferred and ESBL production was not determinable by the Etest. The antibiograms of all seven isolates were in keeping with combined L-1 and L-2 production, even showing sensitivity to ticarcillin/clavulanic acid by disk diffusion testing. Apparently ticarcillin is only a weak substrate for, or is stable to, the L-1 ß-lactamase and clavulanic acid protects ticarcillin against the L-2 enzyme [303]. However it has been shown that even laboratory mutants lacking both L-1 and L-2 enzymes remain resistant to many ß-lactams at breakpoint [304]. Perhaps, at worst, the selection pressures of SPEAR in general, but not specifically the cefotaxime component, contributed to the isolation of five *Stenotrophomonas maltophilia* isolates from three patients who were not temporally or epidemiologically related.

Chapter 5 Conclusions and Recommendations

During the course of two years and ten months 173 isolates of AGNB from the surveillance flora of 92 patients, 26 medical and 66 surgical in a general medical/surgical ITU, were found to be resistant or to show reduced sensitivity to cefotaxime. Another two isolates from one of these patients did not show pharmacological resistance to cefotaxime but they produced ESBLs which caused resistance to multiple ß-lactams and would have compromised the *in vivo* effectiveness of cefotaxime.

5.1 Percentage of patients carrying cefotaxime-resistant AGNB

As 969 patients were treated in the ITU during the time that the isolates were being collected, 9% (92 of 969) of patients carried AGNB which had reduced susceptibility or resistance to cefotaxime. This represented a substantial pool of resistant organisms with the potential for dissemination.

5.2 Cefotaxime resistance unlikely to be related to SPEAR

In 30 of the 92 patients carrying such AGNB, the organisms were grown only from surveillance flora sampled on the patients' first day in ITU. As these samples were obtained within four hours of admission to ITU and before SPEAR had been started it was unlikely that the reduced susceptibility to cefotaxime was related to SPEAR in the isolates from these patients.

5.3 Cefotaxime resistance directly related to SPEAR

In another 38 of the 92 patients who carried AGNB showing reduced susceptibility or resistance to cefotaxime, the organisms were isolated from samples taken on the patients' second and subsequent days in ITU, but not on the first. There was evidence of chromosomal AmpC ß-lactamase derepression in the AGNB

(*Citrobacter freundii* four isolates, *Enterobacter cloacae* 21 isolates, *Morganella morganii* two isolates, *Pseudomonas aeruginosa* three isolates) of 18 of these 38 patients, one of whom (patient 14) had received cefotaxime therapy during the four days preceding his admission to ITU. Therefore chromosomal AmpC enzyme derepression directly related to SPEAR was inferred in the AGNB of 17 patients i.e. 1.8% of patients treated in the ITU over two years and 10 months.

5.4 Cefotaxime resistance indirectly related to SPEAR

In the remaining 21 patients of the above group (including the patient from whom the ESBL-producing AGNB were grown) and in a further 24 patients from whom AGNB with reduced susceptibility or resistance to cefotaxime were isolated on their first and subsequent days in ITU, it is likely that the general selection pressures imposed by SPEAR contributed to their continuing colonization with such organisms. Therefore in 45 patients the cefotaxime-resistance of their AGNB was inferred to be indirectly related to SPEAR.

5.5 Chromosomal ß-lactamases

There was evidence of chromosomal AmpC ß-lactamase derepression in 65 of the 175 SPEAR isolates. It was thought to be directly related to SPEAR in thirty (24%) of the 124 isolates belonging to the ß-lactamase-inducible species (*Enterobacter*, *Citrobacter*, *Morganella*, *Pseudomonas*) but there was no evidence of the emergence of an epidemic strain.

5.6 ESBLs

Strong evidence for ESBL-production was shown in two isolates of *Klebsiella pneumoniae* from one patient only (patient 81), although it was a possibility in two *Acinetobacter* isolates from the same patient. However this study highlighted the difficulties in predicting ß-lactamase identity in certain isolates of the ß-lactamase-

inducible species, even with the knowledge of their antibiograms, plasmid profiles and the pI values of their enzymes. ESBL detection in these organisms poses an even greater problem than in *Klebsiella* species and in *Escherichia coli*. Presently the only way of conclusively identifying an ESBL is to sequence the appropriate gene which is usually plasmid-borne. However transposon and chromosomal integration are thought to be common, with resultant wide dissemination and stability of resistance even in the absence of selective pressure. ESBL-production was first detected in the AGNB from patient 81 on her sixth day in the ITU. Although ceftazidime therapy had been instituted 16 days prior to her admission to ITU it is probable that ESBL selection was at least indirectly related to SPEAR.

5.7 Practical recommendations for the future

- **Microbiological screening must continue**

It is essential that microbiological screening of surveillance flora of ITU patients should continue in order to identify resistance trends and the emergence of epidemic strains which may herald resistance in clinical isolates. A dedicated ITU bench and data-base showing results of both screening and clinical samples for all patients each day 'at a glance' are helpful in expediting this process. All isolates should be identified to species level and antibiotic susceptibility tests recorded quantitatively i.e. diameters of zones of inhibition should be measured and recorded. Staff must be alert to 'suspicious' patterns of resistance such as combined third-generation cephalosporin and aminoglycoside resistance and ceftazidime resistance in an apparently cefotaxime-sensitive isolate. The limitations of pharmacological breakpoints for resistance used by the NCCLS should be recognized and the concept of biological resistance understood.

- **Cefotaxime alone should not be used to screen for ESBLs**

Isolates should be tested initially for sensitivity to ceftazidime as well as cefotaxime. Most ESBLs show resistance to ceftazidime but many are apparently sensitive to cefotaxime as was the case in the present study. If cefotaxime alone is used many ESBL-producers will be missed. Etests for ESBL production (using both cefotaxime and ceftazidime) might then be performed on resistant isolates. However none of these tests is discriminatory for most ESBL-producing members of the ß-lactamase-inducible species. Epidemiological vigilance may be the best practical option for their detection so far.

- **Rigorous standards of hand-washing and hygiene are the mainstay of infection control**

That there was no evidence of dissemination of an epidemic strain can largely be attributed to the high standards of hand-washing and hygiene in this ITU.

- **SPEAR should continue**

The final conclusion is that SPEAR should continue at this location, but only within the context of intensive microbiological monitoring and rigorous hygienic precautions. The guiding principle must always be "primum non nocere" - "first of all to do no harm". The challenge is to know when harm is being done.

References

[1] Polk Jr HC, Fry D & Flint JLM. Dissemination and causes of infection. Surgical Clinics of North America 1976; **56**: 817-829.

[2] Watt I & Ledingham IMcA. Mortality amongst multiple trauma patients admitted to an intensive therapy unit. Anaesthesia 1984; **39**: 973-981.

[3] Stoutenbeek CP, van Saene HKF, Miranda DR & Zandstra DF. The effect of selective decontamination of the digestive tract on colonisation and infection rate in multiple trauma patients. Intensive Care Medicine 1984; **10**: 185-192.

[4] van der Waaij D, Berghuis-deVries JM & Lekkerkerk-van der Wees JEC. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. Journal of Hygiene Cambridge 1971; **69**: 405-411.

[5] van der Waaij D, Berghuis JM & Lekkerkerk JEC. Colonization resistance of the digestive tract of mice during systemic antibiotic treatment. Journal of Hygiene Cambridge 1972; **70**: 605-610.

[6] Miranda DR, van Saene HKF, Stoutenbeek ChP & Zandstra DF. Environment and costs in surgical intensive care unit. The implications of selective decontamination of the digestive tract (SDD). Acta Anaesthesiologica Belgica 1983; **34**: 223-232.

[7] Ledingham IMcA, Alcock SR, Eastaway AT, McDonald JC, McKay IC & Ramsay G. Triple regimen of selective decontamination of the digestive tract, systemic cefotaxime, and microbiological surveillance for the prevention of acquired infection in intensive care. Lancet 1988; **i**: 785-790.

[8] Editorial. Microbial selective decontamination in intensive care patients. Lancet 1988; **i**: 803-804.

[9] Selective Decontamination of the Digestive Tract Trialists' Collaborative Group. Meta-analysis of randomised controlled trials of selective decontamination of the digestive tract. British Medical Journal 1993; **307**: 525-532.

[10] Bartlett JG. Selective decontamination of the digestive tract. Critical Care Medicine 1995; **23**: 613-615.

[11] D'Amico R, Pifferi S, Leonetti C, Torri V, Tinazzi A & Liberati A. Effectiveness of antibiotic prophylaxis in critically ill adult patients: systematic review of randomised control trials. British Medical Journal 1998; **316**: 1275-1285.

[12] Nathens AB & Marshall JC. Selective decontamination of the digestive tract in surgical patients: a systematic review of the evidence. Archives of Surgery 1999; **134**: 170-176.

[13] Lambert HP & O'Grady FW. Antibiotic and Chemotherapy. 6th ed. 1992; Churchill Livingstone, Edinburgh.

[14] Livermore DM. Clinical significance of beta-lactamase induction and stable derepression in Gram-negative rods. European Journal of Clinical Microbiology and Infectious Diseases 1987; **6**: 439-445.

[15] Follath F, Costa E, Thommen A, Frei R, Burdeska A & Meyer J. Clinical consequences of development of resistance to third generation cephalosporins. European Journal of Clinical Microbiology and Infectious Diseases 1987; **6**: 446-450.

[16] Sanders Jr WE & Sanders CC. Inducible ß-lactamases: clinical and epidemiologic implications for use of newer cephalosporins. Reviews of Infectious Diseases 1988; **10**: 830-837.

[17] Chow JW, Fine MJ, Shlaes DM, Quinn JP, Hooper DC, Johnson MP, Ramphal R, Wagener MM, Miyashiro DK & Yu VL. *Enterobacter* bacteraemia: clinical features and emergence of antibiotic resistance during therapy. Annals of Internal Medicine 1991; **115**: 585-590.

[18] Ehrhardt AF & Sanders CC. ß-Lactam resistance amongst *Enterobacter* species. Journal of Antimicrobial Chemotherapy 1993; **32**: 1-11.

[19] Sirot D, Sirot J, Labia R, Morand A, Courvalin P, Darfeuille-Michaud A, Perroux R & Cluzel R. Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel beta-lactamase. Journal of Antimicrobial Chemotherapy 1987; **20**: 323-334.

[20] Sirot J, Chanal C, Petit A, Labia R & Gerbaud G. *Klebsiella pneumoniae* and other enterobacteriaceae producing novel plasmid-mediated ß-lactamases markedly active against third-generation cephalosporins: epidemiologic studies. Reviews of Infectious Diseases 1988; **10**: 850-859.

[21] Quinn JP, Miyashiro D, Sahm D, Flamm R & Bush K. Novel plasmid-mediated ß-lactamase (TEM 10) conferring selective resistance to ceftazidime and aztreonam in clinical isolates of *Klebsiella pneumoniae*. Antimicrobial Agents and Chemotherapy 1989; **33**: 1451-1456.

[22] Petit A, Gerbaud G, Sirot D, Courvalin P & Sirot J. Molecular epidemiology of TEM-3 (CTX-1) ß-lactamase. Antimicrobial Agents and Chemotherapy 1990; **34**: 219-224.

[23] De Champs C, Sirot D, Chanal C, Poupart Marie-C, Dumas Marie-P & Sirot J. Concomitant dissemination of three extended-spectrum ß-lactamases among different Enterobacteriaceae isolated in a French hospital. Journal of Antimicrobial Chemotherapy 1991; **27**: 441-457.

[24] MacKenzie FM & Gould IM. Extended spectrum ß-lactamases. Journal of Infection 1998; **36**: 255-258.

[25] Machiedo GW, LoVerme PJ & McGovern PJ. Patterns of mortality in a surgical intensive care unit. Surgery, Gynecology and Obstetrics 1981; **152**: 757-759.

[26] Daschner F. Nosocomial infections in intensive care units. Intensive Care Medicine 1985; **11**: 284-287.

[27] Gaya H. Infection control in intensive care. British Journal of Anaesthesia 1976; **48**: 9-12.

[28] Thorp JM, Richards WC & Telfer ABM. A survey of infection in an intensive care unit. "Forewarned is forearmed". Anaesthesia 1979; **34**: 643-650.

[29] Hillman KM, Riordan T, O'Farrell SM & Tabaqchali S. Colonisation of the gastric contents in critcally ill patients. Critical Care Medicine 1982; **10**: 444-447.

[30] Kerver AJH, Rommes JH, Mevissen-Verhage EAE, Hulstaert PF, Vos A, Verhoef J & Wittebol P. Colonization and infection in surgical intensive care patients - a prospective study. Intensive Care Medicine 1987; **13**: 347-351.

[31] Donowitz LG, Wenzel RP & Hoyt JW. High risk of hospital acquired infection in the ICU patient. Critical Care Medicine 1982; **10**: 355-357.

[32] Potgieter PD, Linton DM, Oliver S & Forder AA. Nosocomial infections in a respiratory intensive care unit. Critical Care Medicine 1987; **15**: 495-498.

[33] Haley RW, Hooton TM, Culver DH, Stanley RC, Emori TG, Hardison CD, Quade D, Schachtman RH, Schaberg DR Babu V & Schatz GD. Nosocomial infection in U.S. hospitals, 1975-76: Estimated frequency by selected characteristics of patients. American Journal of Medicine 1981; **70**: 947-959.

[34] Northey D, Adess ML, Hartsack JM & Rhoades ER. Microbial surveillance in a surgical Intensive Care Unit. Surgery, Gynecology and Obstetrics 1974; **139**: 321-325.

[35] Wenzel RP, Osterman CA & Hunting KJ. Hospital-acquired infections: II. Infection rates by site, service and common procedures in a university hospital. American journal of Epidemiology 1976; **104**: 645-651.

[36] Johanson WG Jr, Pierce AK, Sanford JP & Thomas GD. Nosocomial respiratory infections with gram-negative bacilli. The significance of colonization of the respiratory tract. Annals of Internal Medicine 1972; **77**: 701-706.

[37] La Force FM. Hospital-acquired Gram-negative rod pneumonias: an overview. American Journal of Medicine 1981; **70**: 664-669.

[38] Brown RB, Hosmer D, Chen HC, Teres D, Sands M, Bradley S, Opitz E, Szwedzinski D & Opalenik D. A comparison of infections in different ICU's within the same hospital. Critical Care Medicine 1985; **13**: 472-475.

[39] Chandrasekar PH, Kruse JA & Mathews MF. Nosocomial infection among patients in different types of intensive care unit at a city hospital. Critical Care Medicine 1986; **14**: 508-510.

[40] Johanson Jr WG, Pierce AK, Sanford JP & Thomas GD. Nosocomial respiratory infections with Gram-negative bacilli. The significance of colonization of the respiratory tract. Annals of Internal Medicine 1972; **77**: 701-706.

[41] Daschner FD, Frey P, Wolff G, Baumann PC & Suter P. Nosocomial infections in intensive care wards: a multicenter prospective study. Intensive Care Medicine 1982; **8**: 5-9.

[42] Unertl K, Ruckdeschel G, Selbmann HK, Jensen U, Forst H, Lenhart FP & Peter K. Prevention of colonization and respiratory infections in long-term ventilated patients by local antimicrobial prophylaxis. Intensive Care Medicine 1987; **13**: 106-113.

[43] Vincent Jean-L, Bihari DH, Suter PM, Bruining HA, White J, Nicolas-Chanoin Marie-H, Wolff M, Spencer RC & Hemmer M. The prevalence of nosocomial infection in intensive care units in Europe. Results of the European Prevalence of Infection in Intensive Care (EPIC) Study. Journal of the American Medical Association 1995; **274**: 639-644.

[44] Snydman DR. Clinical implications of multi-drug resistance in the intensive care unit. Scandinavian Journal of Infectious Diseases 1991; **23** (Suppl. 78): 54-63.

[45] Stoddart JC. The prevention of infection in the intensive care unit. Acta Anaesthesiologica Belgica 183; **34**: 155-156.

[46] Schimpff SC, Young VM, Greene WH, Vermeulen GD, Moody MR & Wiernik PH. Origin of infection in acute nonlymphocytic leukemia. Significance of hospital acquisition of potential pathogens. Annals of Internal Medicine 1972; **77**: 707-714.

[47] Guiot HFL & van Furth R. Partial antibiotic decontamination. British Medical Journal 1977; **1**: 800-802.

[48] Sleijfer DTH, Mulder NH, de Vries-Hospers HG, Fidler V, Nieweg HO, van der Waaij D & van Saene HKF. Infection prevention in granulocytopaenic patients by selective decontamination of the digestive tract. European Journal of Cancer 1980; **16**: 859-869.

[49] Schwartz SN, Dowling JN, Benkovic C, DeQuittner-Buchanan M, Prostko T & Yee RB. Sources of Gram-negative bacilli colonizing the tracheae of intubated patients. Journal of Infectious Diseases 1978; **138**: 227-231.

[50] van Saene HKF, Stoutenbeek CP, Miranda DR & Zandstra DF. A novel approach to infection control in the intensive care unit. Acta Anaesthesiologica Belgica 1983; **34**: 193-208.

[51] Thornton GF & Andriole VT. Bacteriuria during indwelling catheter drainage. Journal of the American Medical Association 1970; **214**: 339-342.

[52] Sanford JP. Infection control in critical care units. Critical Care Medicine 1974; **2**: 211-216.

[53] van Saene HKF, Stoutenbeek CP, Zandstra DF, Gilbertson AA, Murray A & Hart CA. Nosocomial infections in severely traumatised patients: magnitude of problem, pathogenesis, prevention and therapy. Acta Anaesthesiologica Belgica 1987; **38**: 347-353.

[54] Johanson WJ, Pierce AK & Sanford JP. Changing pharyngeal bacterial flora of hospitalised patients. Emergence of Gram-negative bacilli. New England Journal of Medicine 1969; **281**: 1137-1140.

[55] Atherton ST & White DJ. Stomach as source of bacteria colonising respiratory tract during artificial ventilation. Lancet 1978; **ii**: 968-969.

[56] Driks MR, Craven DE, Celli BR, Manning M, Burke RA, Garvin GM, Kunches LM, Farber HW, Wedel SA & McCabe WR. Nosocomial pneumonia in intubated patients given sucralfate as compared with antacids or histamine type 2 blockers. The role of gastric colonization. New England Journal of Medicine 1987; **317**: 1376-1382.

[57] Yoshioka H, Fujita K, Maruyama S & Oka R. Changes in aerobic pharyngeal flora related to antibiotic use and the emergence of Gram-negative bacilli. Clinical Paediatrics 1982; **21**: 460-462.

[58] du Moulin GC, Paterson DG, Hedley-Whyte J & Lisbon A. Aspiration of gastric bacteria in antacid treated patients: a frequent cause of postoperative colonisation in the airway. Lancet 1982; **i**: 242-245.

[59] Tryba M. Risk of acute stress bleeding and nosocomial pneumonia in ventilated intensive care unit patients. American Journal of Medicine 1987; **83** (Suppl. 3B): 117-124.

[60] van der Waaij D & Berghuis-de Vries JM. Selective elimination of *Enterobacteriaceae* species from the digestive tract in mice and monkeys. Journal of Hygiene Cambridge 1974; **72**: 205-211.

[61] van der Waaij D. Selective decontamination of the digestive tract in surgical intensive care units. Intensive Care World 1987; **4**: 44-47.

[62] van der Waaij D, de Vries-Hospers HG & Welling GW. The influence of antibiotics on gut colonization. Journal of Antimicrobial Chemotherapy 1986; **18** (Suppl. C): 155-158.

[63] van der Waaij D. Evidence of immunoregulation of the composition of intestinal microflora and its practical consequences. European Journal of Clinical Microbiology and Infectious Diseases 1988; **7**: 103-106.

[64] Wells CL, Maddaus MA, Jechorek RP & Simmons RL. Role of intestinal anaerobic bacteria in colonization resistance. European Journal of Clinical Microbiology and Infectious Diseases 1988; **7**: 107-113.

[65] van der Waaij D, Berghuis-de Vries JM & Lekkerkerk-van der Wees JEC. Colonization resistance of the digestive tract and the spread of bacteria to the lymphatic organs in mice. Journal of Hygiene Cambridge 1972; **70**: 335-342.

[66] Guiot HFL, van dan Broek PJ, van der Meer JWM & van Furth R. Selective antimicrobial modulation of the intestinal flora of patients with acute non-lymphocytic leukaemia: a double-blind, placebo-controlled study. Journal of Infectious Diseases 1983; **147**: 615-623.

[67] Widmer AF. Infection control and prevention strategies in the ICU. Intensive Care Medicine 1994; **20** (Suppl. 4): S7-S11.

[68] van Saene HKF. Selective decontamination of the digestive tract for critically ill patients: a life saver. British Journal of Intensive Care 1998; **8**: 157.

[69] Thielman NM & Neu HC. Bacterial Cell Wall Inhibitors. In: Brody TM, Larner J & Minneman KP, eds. Human Pharmacology. 3rd ed. 1998; 655-682. Mosby, St Louis, Missouri.

[70] Johnson JR, Woodward RB & Robinson R. The constitution of the penicillins. In: Clarke HT, Johnson JR & Robinson SR, eds. The Chemistry of Penicillin. 1949; 440-454. Princeton University Press, Princeton, New Jersey.

[71] Ghuysen JM. Serine ß-lactamases and penicillin-binding proteins. Annual Review of Microbiology 1991; **45**: 37-67.

[72] Spratt BG. Penicillin-binding proteins and the future of ß-lactam antibiotics. The seventh Fleming lecture. Journal of General Microbiology 1983; **129**: 1247-1260.

[73] Tipper DJ. Mode of action of ß-lactam antibiotics. Pharmacology and Therapeutics 1985; **27**: 1-35.

[74] Waxman DJ, Yocum RR & Strominger JL. Penicillins and cephalosporins are active site-directed acylating agents: evidence in support of the substrate analogue hypothesis. Philosophical Transactions of the Royal Society of London. Series B 1980; **289**: 257-271.

[75] Yocum RR, Rasmussen JR & Strominger JL. The mechanism of action of penicillin: penicillin acylates the active site of *Bacillus stearothermophilus* D-alanine carboxypeptidase. Journal of Biological Chemistry 1980; **255**: 3977-3986.

[76] Kitano K & Tomasz A. Triggering of autolytic cell wall degradation in *Escherichia coli* by beta-lactam antibiotics. Antimicrobial Agents and Chemotherapy 1979; **16**: 838-848.

[77] Handwerger S & Tomasz A. Antibiotic tolerance among clinical isolates of bacteria. Reviews of Infectious Diseases 1985; **7**: 368-386.

[78] Fleming A. On the antibacterial action of cultures of a penicillium with special reference to their use in isolation of *B. influenzae*. British Journal of Experimental Pathology 1929; **10**: 226-236.

[79] Clarke HT, Johnson JR & Robinson SR. Brief history of the chemical study of penicillin. In: Clarke HT, Johnson JR & Robinson SR, eds. The Chemistry of Penicillin. 1949; 3-9. Princeton University Press: Princeton, New Jersey.

[80] Kaczka E & Folkers K. Desthiobenzylpenicillin and other hydrogenolysis products of benzylpenicillin. In: Clarke HT, Johnson JR & Robinson SR, eds. The Chemistry of Penicillin. 1949; 243-268. Princeton University Press, Princeton, New Jersey.

[81] Crowfoot D, Bunn CW, Rogers-Low BW & Turner-Jones A. The X-ray crystallographic investigation of the structure of penicillin. In: Clarke HT, Johnson JR & Robinson SR, eds. The Chemistry of Penicillin. 1949; 310-381. Princeton University Press, Princeton, New Jersey.

[82] Batchelor FR, Doyle FP, Nayler JHC & Rolinson GN. Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. Nature 1959; **183**: 257-258.

[83] Batchelor FR, Chain EB, Richards M & Rolinson GN. 6-Aminopenicillanic acid VI. formation of 6-aminopenicillanic acid from penicillin by enzymic hydrolysis. Proceedings of the Royal Society of London 1961; **B 154**: 522-531.

[84] Abraham EP. Cephalosporins 1945-1986. In: Williams JD, ed. The Cephalosporin Antibiotics. 1987; 1-14. Adis Press, Auckland.

[85] Newton GGF & Abraham EP. Cephalosporin C, a new antibiotic containing sulphur and D-alpha-aminoadipic acid. Nature 1955; **175**: 548.

[86] Rolinson GN. The influence of 6-aminopenicillanic acid on antibiotic development. Journal of Antimicrobial Chemotherapy 1988; **22**: 5-14.

[87] Abraham EP & Newton GGF. The structure of cephalosporin C. Biochemical Journal 1961; **79**: 377-393.

[88] Karchmer AW. Cephalosporins. In: Mandell GL, Bennett JE & Dolin R, eds. Principles and Practice of Infectious Diseases. 4th ed. 1995; vol. 1: 247-264. Churchill Livingstone: New York.

[89] Bucourt R, Bormann D, Heymes R & Perronnet M. Chemistry of cefotaxime. Journal of Antimicrobial Chemotherapy 1980; **6** (Suppl. A): 63-67.

[90] Nikaido H & Nakae T. The outer membrane of gram-negative bacteria. Advances in Microbial Physiology 1979; **20**: 163-250.

[91] Gutmann L, Williamson R & Collatz E. The possible role of porins in antibiotic resistance. Annals of Internal Medicine 1984; **101**: 554-557.

[92] Yoshimura F & Nikaido H. Diffusion of ß-lactam antibiotics through the porin channels of *Escherichia coli* K-12. Antimicrobial Agents and Chemotherapy 1985; **27**: 84-92.

[93] Nikaido H. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrobial Agents and Chemotherapy 1989; **33**: 1831-1836.

[94] Sawai T, Yamaguchi A & Hiruma R. Effect of interaction between outer membrane permeability and ß-lactamase production on resistance to ß-lactam agents in Gram-negative bacteria. Reviews of Infectious Diseases 1988; **10**: 761-764.

[95] Wiedemann B, Kliebe C & Kresken M. The epidemiology of ß-lactamases. Journal of Antimicrobial Chemotherapy 1989; **24** (Suppl. B): 1-22.

[96] Abraham EP & Chain E. An enzyme from bacteria able to destroy penicillin. Nature 1940; **146**: 837.

[97] Abraham EP, Baker W, Boon WR, Calam CT, Carrington HC, Chain E, Florey HW, Freeman GG, Robinson R & Sanders AG. The earlier investigations relating to 2-pentenylpenicillin. In: Clarke HT, Johnson JR & Robinson SR, eds. The Chemistry of Penicillin. 1949; 10-37. Princeton University Press, Princeton, New Jersey.

[98] Medeiros AA. ß-Lactamases. British Medical Bulletin 1984; **40**: 18-27.

[99] Pollock MR. Penicillinase. In: Bayer PD, Lardy H & Myrback K, eds. The Enzymes. 2nd ed. 1960; vol. 4: 269-278. Academic Press: London and New York.

[100] Newton GGF & Abraham EP. Experiments on the degradation of cephalosporin C. Biochemical Journal 1956; **62**: 651-658.

[101] Citri N & Pollock MR. The Biochemistry and Function of ß-Lactamase (penicillinase). Advances in Enzymology 1966; **28**: 237-323.

[102] Kopecko DJ. Specialized genetic recombination systems in bacteria: their involvement in gene expression and evolution. In: Hahn FE & Kersten H, eds. Progress in Molecular and Subcellular Biology. 1980; vol. 7: 135-234. Springer-Verlag: Berlin.

[103] Kopecko DJ. Involvement of specialized recombination in the evolution and expression of bacterial genes. In: Stutgart C & Rozel KR, eds. Plasmids and Transposons. 1980; 165-206. Academic Press, New York.

[104] Datta N. Plasmids as organisms. In: Helinski DR, Cohen SN, Clewell DB, Jackson DA & Hollaender A, eds. Plasmids in Bacteria. 1985; 3-19. Plenum Press, New York.

[105] Mayer KH, Opal SM & Medeiros AA. Mechanisms of antibiotic resistance. In: Mandell GL, Bennett JE & Dolin R, eds. Principles and Practice of Infectious Diseases. 4th ed. 1995; vol. 1: 212-225. Churchill Livingstone: New York.

[106] Nordström K. Replication, incompatibility, and partition. In: Helinski DR, Cohen SN, Clewell DB, Jackson DA & Hollaender A, eds. Plasmids in Bacteria. 1985; 119-123. Plenum Press, New York.

[107] Thompson R. R plasmid transfer. Journal of Antimicrobial Chemotherapy 1986; **18** (Suppl. C): 13-23.

[108] Lederberg J. Cell Genetics and Hereditary Symbiosis. Physiological Reviews 1952; **32**: 403-430.

[109] Lederberg J. Plasmid (1952-1997). Plasmid 1998; **39**: 1-9.

[110] Lederberg J, Cavalli LL & Lederberg EM. Sex compatibility in *Escherichia coli*. Genetics 1952; **37**: 720-730.

[111] Hayes W. Observations on a transmissible agent determining sexual differentation in *Bact. coli*. Journal of General Microbiology 1953; **8**: 72-88.

[112] Clowes RC. Molecular Structure of Bacterial Plasmids. Bacteriological Reviews 1972; **36**: 361-405.

[113] Lavallé R & Jacob F. Sur la sensibilité des épisomes sexuel et colicinogène d'Escherichia coli K12 à la désintégration du radiophosphore. Comptes Rendus de l'Académie des Sciences 1961; **252**: 1678-1680.

[114] Silver S & Ozeki H. Transfer of deoxyribonucleic acid accompanying the transmission of colicinogenic properties by cell mating. Nature 1962; **195**: 873-874.

[115] Watanabe T & Takano T. Episomic resistance factors in *Enterobacteriaceae*. 18. Inactivation of four drug-resistance R factors by the decay of incorporated P^{32}. Medicine and Biology (Tokyo) 1963; **65**: 111-114.

[116] Marmur J, Rownd R, Falkow S, Baron LS, Schildkraut C & Doty P. The nature of intergeneric episomal infection. Proceedings of the National Academy of Sciences of the United States of America 1961; **47**: 972-979.

[117] McClintock B. Chromosome organization and genic expression. Cold Spring Harbor Symposia on Quantitative Biology 1952; **16**: 13-47.

[118] Lupski JR. Molecular mechanisms for transposition of drug resistance genes and other movable genetic elements. Reviews of Infectious Diseases 1987; **9**: 357-368.

[119] Lederberg J & Tatum EL. Gene recombination in *E. coli*. Nature 1946; **158**: 558.

[120] Willetts N. Conjugation. In: Grinsted J & Bennett PM, eds. Plasmid Technology. 2nd ed. 1988; 49-77. Academic Press, London.

[121] Bradley DE. Morphological and serological relationships of conjugative pili. Plasmid 1980; **4**: 155-169.

[122] Summers DK. The Biology of Plasmids. 1997; Blackwell Science, Oxford.

[123] Willetts NS. Location of the origin of transfer of the sex factor F. Journal of Bacteriology 1972; **112**: 773-778.

[124] Ochiai K, Yamanaka T, Kimura K & Sawada O. Studies on inheritance of drug resistance between *Shigella* strains and *Escherichia coli* strains. Nippon Iji Shimpo 1959; **1861**: 34-46.

[125] Akiba T, Koyama K, Ishiki Y, Kimura S & Fukushima T. On the mechanism of the development of multiple-drug-resistant clones of Shigella. Japanese Journal of Microbiology 1960; **4**: 219-227.

[126] Mitsuhashi S, Harada K & Hashimoto H. Multiple resistance of enteric bacteria and transmission of drug-resistance to other strains by mixed cultivation. Japanese Journal of Experimental Medicine 1960; **30**: 179-184.

[127] Watanabe T & Fukasawa T. Episome-mediated transfer of drug resistance in Enterobacteriaceae. I. Transfer of resistance factors by conjugation. Journal of Bacteriology 1961; **81**: 669-678.

[128] Watanabe T. Infectious heredity of multiple drug resistance in bacteria. Bacteriological Reviews 1963; **27**: 87-115.

[129] Datta N. Transmissible drug resistance in an epidemic strain of *Salmonella typhimurium*. Journal of Hygiene Cambridge 1962; **60**: 301-310.

[130] Lebek G. Über die entstehung mehrfachresistenter Salmonellen. Ein experimenteller beitrag. Zentralblatt fur Bakteriologie. 1. Abt. Originale. A: Medizinische Mikrobiologie, Infektionskrankheiten und Parasitiologie 1963; **188**: 494-505.

[131] Datta N. Infectious drug resistance. British Medical Bulletin 1965; **21**: 254-259.

[132] Akiba T. Mechanism of development of resistance in shigella. Medicine of Japan in 1959. In: Proceedings of the 15th General Meeting of the Japan Medical Association 5, 299-305.

[133] Mitsuhashi S, Harada K, Kameda M, Suzuki M & Egawa R. On the drug-resistance of enteric bacteria. 3. Transmission of the drug-resistance from *Shigella* to F^- or Hfr strains of *E. coli* K-12. Japanese Journal of Experimental Medicine 1960; **30**: 301-306.

[134] Griffith F. The significance of pneumococcal types. Journal of Hygiene Cambridge 1928; **27**: 113-159.

[135] Rownd R, Nakaya R & Nakamura A. Molecular nature of the drug-resistance factors of the enterobacteriaceae. Journal of Molecular Biology 1966; **17**: 376-393.

[136] Jacob F & Wollman EL. Les épisomes, éléments génétiques ajoutés. Comptes Rendus de l'Académie des Sciences 1958; **247**: 154-156.

[137] Harada K, Suzuki M, Kameda M & Mitsuhashi S. On the drug-resistance of enteric bacteria. 2. Transmission of the drug-resistance among *Enterobacteriaceae*. Japanese Journal of Experimental Medicine 1960; **30**: 289-299.

[138] Baron LS & Falkow S. Genetic transfer of episomes from *Salmonella typhosa* to *Vibrio cholerae*. Genetics 1961; **46**: 849.

[139] Falkow S, Marmur J, Carey WF, Spilman WM & Baron LS. Episomic transfer between *Salmonella typhosa* and *Serratia marcescens*. Genetics 1961; **46**: 703-706.

[140] Ginoza HS & Matney TS. Transmission of a resistance transfer factor from *Escherichia coli* to two species of *Pasteurella*. Journal of Bacteriology 1963; **85**: 1177-1178.

[141] Anderson ES & Datta N. Resistance to penicillins and its transfer in Enterobacteriaceae. Lancet 1965; **i**: 407-409.

[142] Harmon SA & Baldwin JN. Nature of the determinant controlling penicillinase production in *Staphylococcus aureus*. Journal of Bacteriology 1964; **87**: 593-597.

[143] Datta N & Kontomichalou P. Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae. Nature 1965; **208**: 239-241.

[144] Datta N & Richmond MH. The purification and properties of a penicillinase whose synthesis is mediated by an R-Factor in *Escherichia coli*. Biochemical Journal 1966; **98**: 204-209.

[145] Kogut M, Pollock MR & Tridgell EJ. Purification of penicillin-induced penicillinase of *Bacillus cereus* NRRL 569: a comparison of its properties with those of a similarly purified penicillinase produced spontaneously by a constitutive mutant strain. Biochemical Journal 1956; **62**: 391-401.

[146] Pollock MR. Purification and properties of penicillinases from two strains of *Bacillus licheniformis*: a chemical, physicochemical and physiological comparison. Biochemical Journal 1965; **94**: 666-675.

[147] Richmond MH. Purification and properties of the exopenicillinase from *Staphylococcus aureus*. Biochemical Journal 1963; **88**: 452-459.

[148] Richmond MH. Wild-type variant of exopenicillinase from *Staphylococcus aureus*. Biochemical Journal 1965; **94**: 584-593.

[149] Neu HC & Heppel LA. On the surface localization of enzymes in *E. coli*. Biochemical and Biophysical Research Communications 1964; **17**: 215-219.

[150] Ayliffe GAJ. Ampicillin inactivation and sensitivity of coliform bacilli. Journal of General Microbiology 1963; **30**: 339-348.

[151] Fleming PC, Goldner M & Glass DG. Observations on the nature, distribution, and significance of cephalosporinase. Lancet 1963; **1**: 1399-1401.

[152] Richmond MH & Sykes RB. The ß-lactamases of gram-negative bacteria and their possible physiological role. In: Rose AH & Tempest DW, eds. Advances in Microbial Physiology. 1973; vol. 9: 31-88. Academic Press: London.

[153] Jack GW, Sykes RB & Richmond MH. The ß-lactamases of Gram-negative bacteria. Postgraduate Medical Journal 1970; **46** (Supplement): 41-43.

[154] Richmond MH, Jack GW & Sykes RB. The ß-lactamases of Gram-negative bacteria including pseudomonads. Annals of the New York Academy of Sciences 1971; **182**: 243-257.

[155] Sykes RB & Matthew M. The beta-lactamases of Gram-negative bacteria and their rôle in resistance to beta-lactam antibiotics. Journal of Antimicrobial Chemotherapy 1976; **2**: 115-157.

[156] Matthew M, Harris AM, Marshall MJ & Ross GW. The use of analytical isoelectric focusing for detection and identification of beta-lactamases. Journal of General Microbiology 1975; **88**: 169-178.

[157] Matthew M & Harris AM. Identification of beta-lactamases by analytical isoelectric focusing: correlation with bacterial taxonomy. Journal of General Microbiology 1976; **94**: 55-67.

[158] Ambler RP. The structure of ß-lactamases. Philosophical Transactions of the Royal Society of London. Series B 1980; **289**: 321-331.

[159] Jaurin B & Grundström T. *ampC* cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of ß-lactamases of the penicillinase type. Proceedings of the National Academy of Sciences of the United States of America 1981; **78**: 4897-4901.

[160] Huovinen P, Huovinen S & Jacoby GA. Sequence of PSE-2 ß-lactamase. Antimicrobial Agents and Chemotherapy 1988; **32**: 134-136.

[161] Ouellette M, Bissonnette L & Roy PH. Precise insertion of antibiotic resistance determinants into Tn*21*-like transposons: Nucleotide sequence of the OXA-1 ß-lactamase gene. Proceedings of the National Academy of Sciences of the United States of America 1987; **84**: 7378-7382.

[162] Pollock MR. Origin and function of penicillinase: a problem in biochemical evolution. British Medical Journal 1967; **4**: 71-77.

[163] Yocum RR, Waxman DL, Rasmussen JR & Strominger JL. Mechanism of penicillin action: penicillin and substrate bind covalently to the same active site serine in two bacterial D-alanine carboxypeptidases. Proceedings of the National Academy of Sciences of the United States of America 1979; **76**: 2730-2734.

[164] Waxman DJ, Amanuma H & Strominger JL. Amino acid sequence homologies between *Escherichia coli* penicillin-binding protein 5 and class A ß-lactamases. FEBS Letters 1982; **139**: 159-163.

[165] Bush K. Recent developments in ß-lactamase research and their implications for the future. Reviews of Infectious Diseases 1988; **10**: 681-690.

[166] Bush K. Characterization of ß-lactamases. Antimicrobial Agents and Chemotherapy 1989; **33**: 259-263.

[167] Bush K. Classification of ß-lactamases: Groups 1, 2a, 2b, and 2b'. Antimicrobial Agents and Chemotherapy 1989; **33**: 264-270.

[168] Bush K. Classification of ß-lactamases: Groups 2c, 2d, 2e, 3, and 4. Antimicrobial Agents and Chemotherapy 1989; **33**: 271-276.

[169] Bush K, Jacoby GA & Medeiros AA. Minireview. A functional classification scheme for ß-lactamases and its correlation with molecular structure. Antimicrobial Agents and Chemotherapy 1995; **39**: 1211-1233.

[170] Livermore DM. ß-Lactamases in laboratory and clinical resistance. Clinical Microbiology Reviews 1995; **8**: 557-584.

[171] Waley SG. ß-Lactamase: mechanism of action. In: Page MI, ed. The Chemistry of ß-Lactams. 1992; 198-228. Blackie Academic, Glasgow.

[172] Normark S, Grundström T & Bergström S. Susceptibility to penicillins and cephalosporins in ß-lactamase producing strains of *E. coli* and relative amount of ß-lactamase produced from these strains. Scandinavian Journal of Infectious Diseases 1980; **12** (Suppl. 25): 23-29.

[173] Sykes RB & Matthew M. Detection, assay and immunology of ß-lactamases. In: Hamilton-Miller JMT & Smith JT, eds. Beta-lactamases. 1979; 17-49. Academic Press, London.

[174] Opal SM, Mayer KH & Medeiros AA. Mechanisms of Bacterial Antibiotic Resistance. In: Mandell GL, Bennett JE & Dolin R, eds. Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases. 5th ed. 2000; vol. 1: 236-253. Churchill Livingstone: Philadelphia.

[175] Matthew M. Plasmid-mediated ß-lactamases of Gram-negative bacteria: properties and distribution. Journal of Antimicrobial Chemotherapy 1979; **5**: 349-358.

[176] Livermore DM, Pitt TL, Jones CS, Crees-Morris JA & Williams RJ. PSE-4 ß-lactamase: a serotype-specific enzyme in *Pseudomonas aeruginosa*. Journal of Medical Microbiology 1985; **19**: 45-53.

[177] Medeiros AA. Evolution and dissemination of ß-lactamases accelerated by generations of ß-lactam antibiotics. Clinical Infectious Diseases 1997; **24** (Suppl. 1): S19-S45.

[178] Korfmann G, Kliebe C & Wiedemann B. Beta-lactam antibiotics and selection of resistance: speculation on the evolution of R-plasmids. Journal of Antimicrobial Chemotherapy 1986; **18** (Suppl. C): 113-121.

[179] Kontomichalou P, Mitani M & Clowes RC. Circular R-factor molecules controlling penicillinase synthesis, replicating in *Escherichia coli* under either relaxed or stringent control. Journal of Bacteriology 1970; **104**: 34-44.

[180] Hedges RW, Datta N, Kontomichalou P & Smith JT. Molecular specificities of R factor-determined beta-lactamases: correlation with plasmid compatibility. Journal of Bacteriology 1974; **117**: 56-62.

[181] Matthew M & Hedges RW. Analytical isoelectric focusing of R factor-determined ß-lactamases: correlation with plasmid compatibility. Journal of Bacteriology 1976; **125**: 713-718.

[182] Meynell E & Datta N. The relation of resistance transfer factors to the F-factor (sex factor) of *Escherichia coli* K12. Genetical Research 1966; **7**: 134-140.

[183] Smith JT. R-factor gene expression in Gram-negative bacteria. Journal of General Microbiology 1969; **55**: 109-120.

[184] Dale JW & Smith JT. R-factor-mediated ß-lactamases that hydrolyze oxacillin: evidence for two distinct groups. Journal of Bacteriology 1974; **119**: 351-356.

[185] Matthew M, Hedges RW & Smith JT. Types of ß-lactamase determined by plasmids in Gram-negative bacteria. Journal of Bacteriology 1979; **138**: 657-662.

[186] Pitton JS. Mechanisms of bacterial resistance to antibiotics. In: Adrian RH, ed. Review of Physiology. 1972; vol. 65: 15-93. Springer: Berlin.

[187] Nugent ME & Hedges RW. The nature of the genetic determinant for the SHV-1 ß-lactamase. Molecular and General Genetics 1979; **175**: 239-243.

[188] Knothe H, Shah P, Kremery V, Antal M & Mitsuhashi S. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection 1983; **11**: 315-317.

[189] Kliebe C, Nies BA, Meyer JF, Tolxdorf-Neutzling RM & Wiedemann B. Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. Antimicrobial Agents and Chemotherapy 1985; **28**: 302-307.

[190] Jacoby GA & Medeiros AA. Minireview: More extended-spectrum ß-lactamases. Antimicrobial Agents and Chemotherapy 1991; **35**: 1697-1704.

[191] Medeiros AA. Nosocomial outbreaks of multiresistant bacteria: extended-spectrum beta-lactamases have arrived in North America. Annals of Internal Medicine 1993; **119**: 428-430.

[192] National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Disk Susceptibility Tests. Approved Standard. NCCLS document M2-A5. 5th ed. 1993; NCCLS, Villanova, Pennsylvania.

[193] Thornsberry C. Antimicrobial susceptibility testing: general considerations. In: Balows A, Hausler Jr WJ, Herrmann KL, Isenberg HD & Shadomy HJ, eds. Manual of Clinical Microbiology. 5th ed. 1991; 1059-1064. American Society for Microbiology, Washington, DC.

[194] National Committee for Clinical Laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standard. NCCLS document M7-A3. 3rd ed. 1993; NCCLS, Villanova, Pennsylvania.

[195] Madigan MT, Martinko JM & Parker J. Biology of Microorganisms. 8th ed. 1997; Prentice-Hall, New Jersey.

[196] Brown MRW & Richards RME. The effects of EDTA on the resistance of *Pseudomonas aeruginosa* to antibacterial agents. Nature 1965; **207**: 1391-1393.

[197] Russell AD. Ethylenediaminetetra-acetic acid. In: Hugo W, ed. Inhibition and Destruction of the Microbial Cell. 1971; 209-223. Academic Press, London.

[198] Hardy KG. Purification of bacterial plasmids. In: Hardy KG, ed. Plasmids-a practical approach. 1987; 1-6. IRL Press, Oxford.

[199] Darnell J, Lodish H & Baltimore D. Molecular Cell Biology. 1986; Scientific American Books, New York.

[200] Grinsted J & Bennett PM. Preparation and Electrophoresis of Plasmid DNA. In: Grinsted J & Bennett PM, eds. Plasmid Technology. 2nd ed. 1990; 129-142. Academic Press, London.

[201] Brown TA. Gene Cloning: an introduction. 1986; Van Nostrand Reinhold, London.

[202] Sambrook J, Fritsch EF & Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. 1989; vol. 1. Cold Spring Harbor Laboratory Press, New York.

[203] Sambrook J, Fritsch EF & Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. 1989; vol. 3. Cold Spring Harbor Laboratory Press, New York.

[204] Bak AL, Christiansen C & Stenderup A. Bacterial genome sizes determined by DNA renaturation studies. Journal of General Microbiology 1970; **64**: 377-380.

[205] Wallace DC & Morowitz HJ. Genome size and evolution. Chromosoma 1973; **40**: 121-126.

[206] Gillis M, De Ley J & De Cleene M. The determination of molecular weight of bacterial genome DNA from renaturation rates. European Journal of Biochemistry 1970; **12**: 143-153.

[207] Kingsbury DT. Estimate of the genome size of various microorganisms. Journal of Bacteriology 1969; **98**: 1400-1401.

[208] The Institute for Genomic Research. TIGR microbial database 1999: a listing of microbial genomes and chromosomes completed and in progress. http://www/tigr.org.

[209] Platt DJ, Taggart J & Heraghty KA. Molecular divergence of the serotype-specific plasmid (pSLT) among strains of *Salmonella typhimurium* of human and veterinary origin and comparison of pSLT with the serotype-specific plasmids of *S. enteritidis* and *S. dublin*. Journal of Medical Microbiology 1988; **27**: 277-284.

[210] Technical Services Sigma-Aldrich Company. Trizma. Sigma Technical Bulletin 1990; **106B**: 1-6.

[211] Morelle G. Plasmid extraction procedure on a miniprep scale. Focus 1989; **11**: 7-8.

[212] Terawaki Y, Takayasu H & Akiba T. Thermosensitive replication of a kanamycin resistance factor. Journal of Bacteriology 1967; **94**: 687-690.

[213] Ishihara M, Kamio Y & Terawaki Y. Cupric ion resistance as a new genetic marker of a temperature sensitive R plasmid, Rts1 in *Escherichia coli*. Biochemical and Biophysical Research Communications 1978; **82**: 75-80.

[214] Public Health Laboratory Service London & Deutsche Sammlung von Mikroorganismenund Zellkulturen GmbH Braunschweig. Joint catalogue of medically important (NCTC) and biotechnologically important (DSM) plasmids, phages, transposons and host strains. 1990; European Resource Centres for Plasmid-Bearing Bacteria, London & Braunschweig.

[215] Threlfall EJ, Rowe B, Ferguson JL & Ward LR. Characterization of plasmids conferring resistance to gentamicin and apramycin in strains of *Salmonella typhimurium* phage type 204c isolated in Britain. Journal of Hygiene Cambridge 1986; **97**: 419-426.

[216] Grinsted J & Bennett PM. Analysis of DNA with Restriction Endonucleases. In: Grinsted J & Bennett PM, eds. Plasmid Technology. 2nd ed. 1990; 143-153. Academic Press, London.

[217] Grinsted J & Bennett PM. Introduction. In: Grinsted J & Bennett PM, eds. Plasmid Technology. 2nd ed. 1990; 1-10. Academic Press, London.

[218] Meyers JA, Sanchez D, Elwell LP & Falkow S. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic Acid. Journal of Bacteriology 1976; **127**: 1529-1537.

[219] Aaij C & Borst P. The Gel Electrophoresis of DNA. Biochimica et Biophysica Acta 1972; **269**: 192-200.

[220] Duerden BI. Advice to contributors (Revised October 1990). Journal of Medical Microbiology 1991; **34**: 1-3.

[221] Willshaw GA, Smith HR & Anderson ES. Application of agarose gel electrophoresis to the characterization of plasmid DNA in drug-resistant enterobacteria. Journal of General Microbiology 1979; **114**: 15-25.

[222] Sealey PG & Southern EM. Gel electrophoresis of DNA. In: Rickwood D & Hames BD, eds. Gel electrophoresis of nucleic acids: a practical approach. 1987; 39-76. IRL Press, Oxford.

[223] Sharp PA, Sugden B & Sambrook J. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry 1973; **12**: 3055-3063.

[224] Microsoft Corporation. Microsoft Excel Function Reference. 1992; Microsoft Corporation, Redmond.

[225] Anhalt JP & Washington II JA. Appendix 1. Preparation and storage of antimicrobial solutions. In: Balows A, Hausler Jr WJ, Herrmann KL, Isenberg HD & Shadomy HJ, eds. Manual of Clinical Microbiology. Fifth ed. 1991; 1199-1200. American Society for Microbiology, Washington DC.

[226] Wolfson JS & Hooper DC. The fluoroquinolones: Structures, mechanisms of action and resistance, and spectra of activity in vitro. Antimicrobial Agents and Chemotherapy 1985; **28**: 581-586.

[227] Fisher LM, Hopewell R, Oram M & Sreedharan S. The molecular basis of quinolone action and resistance. In: Neu HC, ed. New Antibacterial Strategies. 1990; 177-190. Churchill Livingstone, New York.

[228] Livermore DM. British Society for Antimicrobial Chemotherapy ß-Lactamase Workshop Manual. 1993; Department of Medical Microbiology, London Hospital Medical College, London.

[229] O'Callaghan CH, Morris A, Kirby SM & Shingler AH. Novel method for detection of beta-lactamases by using a chromogenic cephalosporin substrate. Antimicrobial Agents and Chemotherapy 1972; **1**: 283-288.

[230] Curtis NAC, Eisenstadt RL, Rudd C & White AJ. Inducible Type I beta-lactamases of Gram-negative bacteria and resistance to beta-lactam antibiotics. Journal of Antimicrobial Chemotherapy 1986; **17**: 51-61.

[231] Haglund H. Isoelectric focusing in natural pH gradients - a technique of growing importance for fractionation and characterisation of proteins. Science Tools 1967; **14**: 17-23.

[232] Menday AP & Tybring L. Mecillinam. In: Reeves DS, Phillips I, Williams JD & Wise R, eds. Laboratory Methods in Antimicrobial Chemotherapy. 1978; 79-81. Churchill Livingstone, Edinburgh.

[233] Fuchs PC, Barry AL, Thornsberry C & Jones RN. Interpretive criteria for temocillin disk diffusion susceptibility testing. European Journal of Clinical Microbiology 1985; **4**: 30-33.

[234] Zeneca Pharmaceuticals. The Meropenem Laboratory Manual. 4th ed. 1994; Zeneca Pharmaceuticals, Macclesfield, Cheshire.

[235] Rochelle PA, Fry JC, Day MJ & Bale MJ. An accurate method for estimating sizes of small and large plasmids and DNA fragments by gel electrophoresis. Journal of General Microbiology 1985; **132** (Pt 1): 53-59.

[236] Platt DJ, Chesham JS, Brown DJ, Kraft CA & Taggart J. Restriction enzyme fingerprinting of enterobacterial plasmids: a simple strategy with wide application. Journal of Hygiene Cambridge 1986; **97**: 205-210.

[237] Bradley DE, Taylor DE & Cohen DR. Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K12. Journal of Bacteriology 1980; **143**: 1466-1470.

[238] Platt DJ & Sommerville JS. A simple method for the detection of resistance plasmids in *Serratia* species by transfer to members of the genus *Enterobacter*. Journal of Antimicrobial Chemotherapy 1981; **8**: 145-152.

[239] Hibbert-Rogers LCF, Heritage J, Gascoyne-Binzi DM, Hawkey PM, Todd N, Lewis IJ & Bailey C. Molecular epidemiology of ceftazidime resistant Enterobacteriaceae from patients on a paediatric oncology ward. Journal of Antimicrobial Chemotherapy 1995; **36**: 65-82.

[240] Liu PYF, Gur D, Hall LMC & Livermore DM. Survey of the prevalence of ß-lactamases amongst 1000 Gram-negative bacilli isolated consecutively at the Royal London Hospital. Journal of Antimicrobial Chemotherapy 1992; **30**: 429-447.

[241] Livermore DM. Mechanisms of resistance to ß-lactam antibiotics. Scandinavian Journal of Infectious Diseases 1991; **23** (Suppl. 78): 7-16.

[242] Wiedemann B. Genetic and biochemical basis of resistance of *Enterobacteriaceae* to beta-lactam antibiotics. Journal of Antimicrobial Chemotherapy 1986; **18** (Suppl. B): 31-38.

[243] Yang Y & Livermore DM. Activity of temocillin and other penicillins against ß-lactamase-inducible and -stably derepressed enterobacteria. Journal of Antimicrobial Chemotherapy 1988; **22**: 299-306.

[244] Bergogne-Bérézin E & Joly-Guillou ML. Antibiotic resistance mechanisms in *Acinetobacter*. In: Towner KJ, Bergogne-Bérézin E & Fewson CA, eds. The Biology of *Acinetobacter*: Taxonomy, Clinical Importance, Molecular Biology, Physiology, Industrial Relevance. 1991; 83-115. Plenum Press, New York.

[245] Goldstein GW, Labigne-Roussel A, Gerbaud G, Carlier C, Collatz E & Courvalin P. Transferable plasmid mediated antibiotic resistance in Acinetobacter. Plasmid 1983; **10**: 138-147.

[246] Phillips I, King A, Shannon K & Warren C. SQ 26.776: *in vitro* antibacterial activity and susceptibility to beta-lactamases. Journal of Antimicrobial Chemotherapy 1981; **8** (Suppl. E): 103-110.

[247] Bergogne-Bérézin E & Joly-Guillou ML. Comparative activity of imipenem, ceftazidime and cefotaxime against *Acinetobacter calcoaceticus*. Journal of Antimicrobial Chemotherapy 1986; **18** (Suppl. E): 35-39.

[248] Joly-Guillou ML, Vallee E, Bergogne-Bérézin E & Philippon A. Distribution of beta-lactamases and phenotype analysis in clinical strains of *Acinetobacter calcoaceticus*. Journal of Antimicrobial Chemotherapy 1988; **22**: 597-604.

[249] Bauernfeind A. Classification of beta-lactamases. Reviews of Infectious Diseases 1986; **8** (Suppl. 5): S470-S481.

[250] Joly-Guillou ML, Bergogne-Bérézin E & Moreau N. Enzymatic resistance to beta-lactams and aminoglycosides. Journal of Antimicrobial Chemotherapy 1987; **20**: 773-776.

[251] Morohoshi T & Saito T. Beta-lactamase and beta-lactam antibiotic resistance in *Acinetobacter anitratus* (syn *A. calcoaceticus*). Journal of Antibiotics 1977; **30**: 969-973.

[252] Sawai T, Kanno M & Tsukamoto K. Characterisation of eight beta-lactamases of gram-negative bacteria. Journal of Bacteriology 1982; **152**: 567-571.

[253] Hood J & Amyes SGB. The chromosomal ß-lactamases of the genus *Acinetobacter*: enzymes which challenge our imagination. In: Towner KJ, Bergogne-Bérézin E & Fewson CA, eds. The Biology of *Acinetobacter*: Taxonomy, Clinical Importance, Molecular Biology, Physiology, Industrial Relevance. 1991; 117-132. Plenum Press, New York.

[254] Kucers A, Crowe SM, Grayson ML & Hoy JF. The Use of Antibiotics. 5th ed. 1997; Butterworth-Heinemann, Oxford.

[255] Tajima M, Takenouchi Y, Sugawara S, Inoue M & Mitsuhashi S. Purification and properties of chromosomally mediated ß-lactamase from *Citrobacter freundii* GN7391. Journal of General Microbiology 1980; **221**: 449-456.

[256] Seeberg AH, Tolxdorff-Neutzling RM & Wiedemann B. Chromosomal ß-lactamases of *Enterobacter cloacae* are responsible for resistance to third-generation cephalosporins. Antimicrobial Agents and Chemotherapy 1983; **23**: 918-925.

[257] Then RL, Charnas RL, Kocher HP, Manneberg M, Röthlisberger U & Stocker J. Biochemical characterization of type A and type B ß-lactamase from *Enterobacter cloacae*. Reviews of Infectious Diseases 1988; **10**: 714-720.

[258] Cartwright SJ & Waley SG. Purification of ß-lactamases by affinity chromatography on phenylboronic acid-agarose. Biochemical Journal 1984; **221**: 505-512.

[259] Bush K, Tanaka SK, Bonner DP & Sykes RB. Resistance caused by decreased penetration of ß-lactam antibiotics into *Enterobacter cloacae*. Antimicrobial Agents and Chemotherapy 1985; **27**: 555-560.

[260] Guerin S, Paradis F & Guay R. Cloning and characterization of chromosomally encoded cephalosporinase gene of *Enterobacter cloacae*. Canadian Journal of Microbiology 1986; **32**: 301-309.

[261] Then RL & Angehrn P. Multiply resistant mutants of *Enterobacter cloacae* selected by ß-lactam antibiotics. Antimicrobial Agents and Chemotherapy 1986; **30**: 684-688.

[262] Sanders CC. Novel resistance selected by the new expanded-spectrum cephalosporins: a concern. Journal of Infectious Diseases 1983; **147**: 585-589.

[263] European Study Group on Antibiotic Resistance. Incidence of inducible beta-lactamases in Gram-negative septicaemia isolates from twenty-nine European laboratories. European Journal of Clinical Microbiology and Infectious Diseases 1987; **6**: 460-466.

[264] Kitzis MD, Billot-Klein D, Goldstein FW, Williamson R, Tran Van Nhieu G, Carlet J, Acar JF & Gutmann L. Dissemination of the novel plasmid-mediated ß-lactamase CTX-1, which confers resistance to broad-spectrum cephalosporins,and its inhibition by ß-lactamase inhibitors. Antimicrobial Agents and Chemotherapy 1988; **32**: 9-14.

[265] Philippon A, Labia R & Jacoby G. Extended-spectrum ß-lactamases. Antimicrobial Agents and Chemotherapy 1989; **33**: 1131-1136.

[266] D'Agata E, Venkataraman L, DeGirolami P, Weigel L, Samore M & Tenover F. The molecular and clinical epidemiology of Enterobacteriaceae producing extended-spectrum ß-lactamase in a tertiary care hospital. Journal of Infection 1998; **36**: 279-285.

[267] Pangon B, Bizet C, Pichon F, Phillipon A, Regnier B, Bure A & Gutmann L. *In vivo* selection of a cephamycin resistant porin mutant of a CTX-1 ß-lactamase producing strain of *Klebsiella pneumoniae*. Journal of Infectious Diseases 1989; **159**: 1005-1006.

[268] Sanders CC & Sanders Jr WE. Microbial resistance to newer generation ß-lactam antibiotics: clinical and laboratory implications. Journal of Infectious Diseases 1985; **151**: 399-406.

[269] Mellencamp MA, Roccaforte JS, Preheim LC, Sanders CC, Anene CA & Bittner MJ. Isolation of *Enterobacter aerogenes* susceptible to ß-lactam antibiotics despite

high level beta-lactamase production. European Journal of Clinical Microbiology and Infectious Diseases 1990; **9**: 827-830.

[270] Sanders CC. ß-Lactamases of Gram-negative bacteria: new challenges for new drugs. Clinical Infectious Diseases 1992; **14**: 1089-1099.

[271] Preheim LC, Penn RG, Sanders CC, Goering RV & Giger DK. Emergence of resistance to ß-lactam and aminoglycoside antibiotics during moxalactam therapy of *Pseudomonas aeruginosa* infections. Antimicrobial Agents and Chemotherapy 1982; **22**: 1037-1041.

[272] Piddock LCJ, Walters RN, Jin Y-F, Turner HL, Gascoyne-Binzi DM & Hawkey PM. Prevalence and mechanism of resistance to 'third-generation' cephalosporins in clinically relevant isolates of Enterobacteriaceae from 43 hospitals in the UK, 1990-1991. Journal of Antimicrobial Chemotherapy 1997; **39**: 177-187.

[273] Sanders CC & Sanders WE. ß-Lactam resistance in Gram-negative bacteria: global trends and clinical impact. Clinical Infectious Diseases 1992; **15**: 824-839.

[274] Blasquez J, Baquero M-R, Canton I, Alos I & Baquero F. Characterization of a new TEM-type ß-lactamase resistant to clavulanate, sulbactam and tazobactam. Antimicrobial Agents and Chemotherapy 1993; **37**: 2059-2063.

[275] Thomson CJ & Amyes SGB. TRC-1: emergence of a clavulanic acid-resistant mutant of TEM-ß-lactamase in a clinical strain. FEMS Microbiology Letters 1992; **91**: 113-118.

[276] Vedel G, Belaaouaj A, Lilly G, Labia R, Philippon A, Nevot P & Paul G. Clinical isolates of *Escherichia coli* producing TRI ß-lactamases: novel TEM enzymes conferring resistance to ß-lactamase inhibitors. Journal of Antimicrobial Chemotherapy 1992; **30**: 449-462.

[277] Zhou XY, Bordon F, Sirot D, Kitzis M-D & Gutmann I. Emergence of clinical isolates of *Escherichia coli* producing TEM-1 derivatives or an OXA-1 ß-lactamase conferring resistance to ß-lactamase inhibitors. Antimicrobial Agents and Chemotherapy 1994; **38**: 1085-1089.

[278] Seetulsingh P, Hall LMC & Livermore DM. Activity of clavulanate combinations against TEM-1 ß-lactamase producing *Escherichia coli* isolates obtained in 1982 and 1989. Journal of Antimicrobial Chemotherapy 1991; **27**: 749-759.

[279] Wu P-J, Shannon K & Phillips I. Effect of hyperproduction of TEM-1 ß-lactamase on in vitro susceptibility of *Escherichia coli* to ß-lactam antibiotics. Antimicrobial Agents and Chemotherapy 1994; **38**: 494-498.

[280] Simpson IN, Plested SJ & Harper PB. Investigation of the ß-lactamase stability of ceftazidime and eight other new cephalosporin antibiotics. Journal of Antimicrobial Chemotherapy 1982; **9**: 357-368.

[281] Bennett PM & Chopra I. Molecular basis of ß-lactamase induction in bacteria. Antimicrobial Agents and Chemotherapy 1993; **37**: 153-158.

[282] Sato K, Fujii R, Okatomo R, Inoue M & Mitsuhashi S. Biochemical properties of ß-lactamase produced by *Flavobacterium odoratum*. Antimicrobial Agents and Chemotherapy 1985; **27**: 612-614.

[283] Jacoby GA & Carreras I. Activities of ß-lactam antibiotics against *Escherichia coli* strains producing extended-spectrum ß-lactamases. Antimicrobial Agents and Chemotherapy 1990; **34**: 858-862.

[284] Payne DJ, Marriott MS & Amyes SGB. Characterisation of a unique ceftazidime-hydrolysing ß-lactamase, TEM-E2. Journal of Medical Microbiology 1990; **32**: 131-134.

[285] Payne DJ & Amyes SGB. Transferable resistance to extended-spectrum ß-lactams: a major threat or minor inconvenience? Journal of Antimicrobial Chemotherapy 1991; **27**: 255-261.

[286] Lucet Jean-C, Chevret S, Decré D, Vanjak D, Macrez A, Bédos Jean-P, Wolff M & Regnier B. Outbreak of multiply resistant enterobacteriaceae in an intensive care unit: epidemiology and risk factors for acquisition. Clinical Infectious Diseases 1995; **22**: 430-436.

[287] Du Bois SK, Marriott MS & Amyes SGB. Review. TEM- and SHV- derived extended spectrum ß-lactamases: relationship between selection, structure and function. Journal of Antimicrobial Chemotherapy 1995; **35**: 7-22.

[288] Baquero F, Cantón R, Martinez-Beltrán J & Bolmström A. The E-test as an epidemiologic tool. Diagnostic Microbiology and Infectious Diseases 1992; **15**: 483-487.

[289] Jacoby GA & Han P. Detection of extended spectrum ß-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli*. Journal of Clinical Microbiology 1996; **34**: 908-911.

[290] Karas JA, Pillay DG, Muckart D & Sturm AW. Treatment failure due to extended-spectrum ß-lactamase. Journal of Antimicrobial Chemotherapy 1996; **37**: 203-204.

[291] Akova M, Yang Y & Livermore DM. Interactions of tazobactam and clavulanate with inducibly and constitutively-expressed class I ß-lactamases. Journal of Antimicrobial Chemotherapy 1990; **25**: 199-208.

[292] Hancock REW & Woodruff W. Roles of porin and ß-lactamase in ß-lactam resistance of *Pseudomonas aeruginosa*. Reviews of Infectious Diseases 1988; **10**: 770-775.

[293] Chen HY, Yuan M & Livermore DM. Mechanisms of resistance to ß-lactam antibiotics amongst *Pseudomonas aeruginosa* isolates collected in the UK in 1993. Journal of Medical Microbiology 1995; **43**: 300-309.

[294] Williams RJ, Livermore DM, Lindridge MA, Said AA & Williams JD. Mechanisms of beta-lactam resistance in British isolates of *Pseudomonas aeruginosa*. Journal of Medical Microbiology 1984; **17**: 283-289.

[295] Shannon KP, King A & Phillips I. development of resistance to beta-lactam antibiotics during therapy of *Pseudomonas aeruginosa* infections. Lancet 1982; **1**: 1466.

[296] Livermore DM, Williams RJ & Williams JD. Comparison of the ß-lactamase stability and the *in-vitro* activity of cefoperazone, cefotaxime, cefsulodin, ceftazidime, moxalactam and ceftriaxone against *Pseudomonas aeruginosa*. Journal of Antimicrobial Chemotherapy 1981; **8**: 323-331.

[297] Livermore DM, Williams RJ, Lindridge MA, Slack RCB & Williams JD. *Pseudomonas aeruginosa* isolates with modified beta-lactamase inducibility: effects on beta-lactam sensitivity. Lancet 1982; **1**: 1466-1467.

[298] Jacoby GA & Matthew M. The distribution of ß-lactamase genes on plasmids found in *Pseudomonas*. Plasmid 1979; **2**: 41-47.

[299] Furth A. The beta-lactamases of *Pseudomonas aeruginosa*. In: Hamilton-Miller JMT & Smith JT, eds. Beta-lactamases. 1979; 403-428. Academic Press, London.

[300] Mathisen GE, Meyer RD, Thompson JM & Finegold SM. Clinical evaluation of moxalactam. Antimicrobial Agents and Chemotherapy 1982; **21**: 780-786.

[301] Bailey RR, Peddie B, Blake E, Bishop V & Reddy J. Cefoperazone in the treatment of severe or complicated infections. Drugs 1981; **22** (Suppl. 1): 76-86.

[302] Bonfiglio G & Livermore DM. Effect of media composition on the susceptibility of *Xanthomonas maltophilia* to ß-lactam antibiotics. Journal of Antimicrobial Chemotherapy 1991; **28**: 837-842.

[303] Pankuch GA, Jacobs MR, Rittenhouse SF & Appelbaum PC. Susceptibilities of 123 strains of *Xanthomonas maltophilia* to eight ß-lactams (including ß-lactam-ß-lactamase inhibitor combinations) and ciprofloxacin tested by five methods. Antimicrobial Agents and Chemotherapy 1994; **38**: 2317-2333.

[304] Akova M, Bonfiglio G & Livermore DM. Susceptibility to ß-lactam antibiotics of mutant strains of *Xanthomonas maltophilia* with high- and low-level constitutive expression of L1 and L2 ß-lactamases. Journal of Medical Microbiology 1991; **35**: 208-213.

[305] Mett H, Rosta S, Schacher B & Frei R. Outer membrane permeability and ß-lactamase content in *Pseudomonas maltophilia* clinical isolates and laboratory mutants. Reviews of Infectious Diseases 1988; **10**: 765-769.

[306] Saino Y, Kobayashi F, Inoue M & Mitsuhashi S. Purification and properties of inducible penicillin ß-lactamase isolated from *Pseudomonas maltophilia*. Antimicrobial Agents and Chemotherapy 1982; **22**: 564-570.

Appendix I : Patients, Isolates and Clinical Details

Patient's No	Age	Isolate No	Isolate	Day	Clinical Summary
1	62	10	*Morganella morganii*	10	Collapse, DVT, PTE. CCF, pneumonia, diabetes, hypothyroidism, cardiac arrest
1	62	9	*Citrobacter freundii*	8	Collapse, DVT, PTE. CCF, pneumonia, diabetes, hypothyroidism, cardiac arrest
2	57	2	*Acinetobacter calco var anitratus*	1	Cardiac arrest, prev frontal lobectomy & XRT, glioblastoma multiforme,
3	67	123	*Acinetobacter calco var lwoffii*	1	Postop Hartman's after dehiscence of sigmoid anastomosis following sigmoid colectomy
4	53	111	*Acinetobacter calco var anitratus*	3	RTA, blunt abd trauma
4	53	112	*Acinetobacter calco var anitratus*	6	RTA, blunt abd trauma
4	53	114	*Acinetobacter calco var anitratus*	8	RTA, blunt abd trauma
5	17	53	*Enterobacter cloacae*	1	Nephrotic syndrome, hypertension, fitting
5	17	54	*Enterobacter cloacae*	3	Nephrotic syndrome, hypertension, fitting
6	64	59	*Pseudomonas aeruginosa*	1	Postop hepatic lobectomy for metastatic colorectal ca. Elective admn for ventilation and monitoring
6	64	60	*Pseudomonas aeruginosa*	3	Postop hepatic lobectomy for metastatic colorectal ca. Elective admn for ventilation and monitoring
7	29	71	*Pseudomonas aeruginosa*	1	Unconscious, pulmonary oedema, infection, sarcoma of spine
8	64	63	*Pseudomonas aeruginosa*	1	Postop Hartman's for perf diverticular abscess and purulent peritonitis
9	63	26	*Enterobacter cloacae*	1	Postop pneumonectomy for bronchogenic ca, readmitted for ventilation after cardiac arrest .
10	69	109	*Pseudomonas aeruginosa*	3	Diminished conscious level, shock team transfer from another hospital, 'flu, discharging ear
10	69	110	*Pseudomonas aeruginosa*	5	Diminished conscious level, shock team transfer from another hospital, 'flu, discharging ear
12	83	19	*Pseudomonas aeruginosa*	1	Ventilation after right cataract extraction and implant
12	83	20	*Pseudomonas aeruginosa*	1	Ventilation after right cataract extraction and implant
13	63	177	*Pseudomonas aeruginosa*	1	Postop after Hartman's procedure L hemicolectomy and repair of bladder
14	37	160	*Pseudomonas aeruginosa*	4	Respiratory distress, severe pneumonia, renal transplant patient
14	37	161	*Enterobacter cloacae*	4	Respiratory distress, severe pneumonia, renal transplant patient
15	58	200	*Escherichia coli*	2	Postop anastomotic breakdown after ant resection rectal ca, multiple PTEs, inf haem, readmit ITU
16	64	36	*Enterobacter cloacae*	1	Postop splenectomy (NHL), readmitted post op resection/anastomosis for ischaemia of small bowel
16	64	37	*Enterobacter cloacae*	1	Postop splenectomy (NHL), readmitted post op resection/anastomosis for ischaemia of small bowel
17	61	72	*Pseudomonas aeruginosa*	1	Postop infected aortic bifurcational graft removal, ischaemic leg, axillo-fem graft
17	61	73	*Pseudomonas aeruginosa*	5	Postop infected aortic bifurcational graft removal, ischaemic leg, axillo-fem graft
18	59	124	*Pseudomonas aeruginosa*	1	Renal & respiratory failure, pleural effusion, chest infection, nephrotic syndrome
18	59	125	*Pseudomonas aeruginosa*	1	Renal & respiratory failure, pleural effusion, chest infection, nephrotic syndrome
19	68	191	*Acinetobacter calco var anitratus*	1	Postop repair anastomosis and Hartman's procedure after ant resection of rectal ca
19	68	195	*Acinetobacter calco var anitratus*	7	Postop repair anastomosis and Hartman's procedure after ant resection of rectal ca
19	68	196	*Acinetobacter calco var anitratus*	4	Postop repair anastomosis and Hartman's procedure after ant resection of rectal ca
20	34	3	*Acinetobacter calco var anitratus*	12	Guillain-Barré syndrome, plasmapheresis, tracheostomy
21	67	158	*Pseudomonas aeruginosa*	5	Postop ventilation hemicolectomy and splenectomy, readmitted ITU
21	67	157	*Morganella morganii*	3	Postop ventilation hemicolectomy and splenectomy, readmitted ITU
22	60	197	*Enterobacter cloacae*	1	Resp failure after endoscopic retrograde cholo-pancreatectomy

Appendix I : Patients, Isolates and Clinical Details

Patient's No	Age	Isolate No	Isolate	Day	Clinical Summary
23	65	106	*Acinetobacter calco var anitratus*	1	Postop cardiac surgery/diabetes/periph vascular disease/severe coronary artery disease
23	65	107	*Acinetobacter calco var anitratus*	3	Postop cardiac surgery/diabetes/periph vascular disease/severe coronary artery disease
23	65	108	*Acinetobacter calco var anitratus*	3	Postop cardiac surgery/diabetes/periph vascular disease/severe coronary artery disease
23	65	105	*Acinetobacter calco var anitratus*	1	Postop cardiac surgery/diabetes/periph vascular disease/severe coronary artery disease
24	73	174	*Enterobacter cloacae*	1	Respiratory arrest, multiple injuries from fall at home
24	73	178	*Enterobacter cloacae*	6	Respiratory arrest, multiple injuries from fall at home
24	73	184	*Enterobacter cloacae*	8	Respiratory arrest, multiple injuries from fall at home
25	57	166	*Pseudomonas aeruginosa*	2	Resp failure postop peritonitis, gastric ca
26	79	128	*Pseudomonas aeruginosa*	1	Shock team transfer from theatre, HD monitoring after postop drainage of abscess
26	79	129	*Pseudomonas aeruginosa*	3	Shock team transfer from theatre, HD monitoring after postop drainage of abscess
27	73	139	*Pseudomonas aeruginosa*	1	Shock team transfer from another hospital, aortic aneurysm
28	62	48	*Pseudomonas aeruginosa*	11	Multiple injuries from RTA
28	62	49	*Pseudomonas aeruginosa*	16	Multiple injuries from RTA
29	74	165	*Citrobacter freundii*	9	Resp failure/COAD/infective exacerbation
30	18	18	*Acinetobacter calco var lwoffii*	1	Postop bilat adrenalectomy & splenectomy, malignant hypertension, bilateral phaeochromocytomas
31	69	146	*Enterobacter cloacae*	1	COAD, ARDS, sepsis, cirrhosis, prev hemicolectomy
31	69	147	*Enterobacter cloacae*	5	COAD, ARDS, sepsis, cirrhosis, prev hemicolectomy
31	69	148	*Enterobacter cloacae*	5	COAD, ARDS, sepsis, cirrhosis, prev hemicolectomy
32	75	6	*Acinetobacter calco var anitratus*	1	Ventilation and monitoring postop subtotal oesophagectomy for ca
33	58	17	*Pseudomonas aeruginosa*	1	Renal failure after elective aorto-femoral bypass at another hospital
33	58	16	*Morganella morganii*	1	Renal failure after elective aorto-femoral bypass at another hospital
34	51	142	*Enterobacter cloacae*	18	Postop after repair of hiatus hernia/tension pneumothorax, further surgery
34	51	141	*Enterobacter cloacae*	18	Postop after repair of hiatus hernia/tension pneumothorax, further surgery
34	51	134	*Enterobacter cloacae*	15	Postop after repair of hiatus hernia/tension pneumothorax, further surgery
34	51	135	*Enterobacter* species	15	Postop after repair of hiatus hernia/tension pneumothorax, further surgery
35	59	82	*Acinetobacter calco var anitratus*	1	Cardiac arrest, COAD, myxoedema, narcolepsy, dyspnoea
36	75	32	*Stenotrophomonas maltophilia*	8	Postop, faecal peritonitis / ruptured diverticulum / resp failure
36	75	28	*Stenotrophomonas maltophilia*	1	Postop, faecal peritonitis / ruptured diverticulum / resp failure
36	75	29	*Stenotrophomonas maltophilia*	1	Postop, faecal peritonitis / ruptured diverticulum / resp failure
36	75	30	*Stenotrophomonas maltophilia*	3	Postop, faecal peritonitis / ruptured diverticulum / resp failure
36	75	31	*Stenotrophomonas maltophilia*	6	Postop, faecal peritonitis / ruptured diverticulum / resp failure
37	74	38	*Pseudomonas aeruginosa*	8	Acute exacerbn of COAD, fall/ fractured ribs 6 wks previously, psoriasis
38	66	153	*Citrobacter freundii*	5	Pancreatitis and respiratory acidosis
39	57	87	*Pseudomonas aeruginosa*	1	Renal transplant, septic arthritis
40	22	126	*Enterobacter cloacae*	3	Postop 2nd laparotomy for abdominal stabbing injuries with sword

Appendix I : Patients, Isolates and Clinical Details

Patient's No	Age	Isolate No	Isolate	Day	Clinical Summary
41	50	50	*Enterobacter cloacae*	2	Postop aorta-SMA graft for SMA gangrene, necrotic bowel, ITU readmission
41	50	51	*Enterobacter cloacae*	2	Postop aorta-SMA graft for SMA gangrene, necrotic bowel, ITU readmission
42	46	57	*Morganella morganii*	15	Post 2nd laparotomy/drainage of necrotic pancreas & pseudocyst, cholecystectomy
42	46	58	*Morganella morganii*	20	Post 2nd laparotomy/drainage of necrotic pancreas & pseudocyst, cholecystectomy
43	64	169	*Hafnia alvei*	1	Postop renal failure, hemicolectomy for caecal perf, faecal peritonitis
43	64	167	*Hafnia alvei*	2	Postop renal failure, hemicolectomy for caecal perf, faecal peritonitis
43	64	168	*Hafnia alvei*	1	Postop renal failure, hemicolectomy for caecal perf, faecal peritonitis
44	47	173	*Acinetobacter calco var anitratus*	1	Pericardiectomy for restrictive cardiomyopathy, sepsis, severe pulmonary oedema, cardiac failure
45	78	56	*Pseudomonas aeruginosa*	1	Renal failure, pyopneumothorax after hernia repair, sepsis
45	78	55	*Pseudomonas aeruginosa*	2	Renal failure, pyopneumothorax after hernia repair, sepsis
46	60	143	*Acinetobacter calco var anitratus*	1	Postop vagotomy& pyloroplasty, polycystic kidney disease
47	60	182	*Pseudomonas aeruginosa*	1	Resp failure/pneumonia, COAD, liver disease
47	60	183	*Pseudomonas aeruginosa*	3	Resp failure/pneumonia, COAD, liver disease
48	75	69	*Pseudomonas species*	5	Postop repair ruptured aortic neurysm
48	75	67	*Pseudomonas* species	1	Postop repair ruptured aortic neurysm
48	75	68	*Pseudomonas* species	3	Postop repair ruptured aortic neurysm
48	75	70	*Pseudomonas* species	5	Postop repair ruptured aortic neurysm
51	79	99	*Stenotrophomonas maltophilia*	2	Postop repair anastomosis/peritonitis/decreasing renal function, hemicolectomy
51	79	100	*Enterobacter cloacae*	1	Postop repair anastomosis/peritonitis/decreasing renal function, hemicolectomy
51	79	101	*Enterobacter cloacae*	2	Postop repair anastomosis/peritonitis/decreasing renal function, hemicolectomy
51	79	102	*Enterobacter cloacae*	4	Postop repair anastomosis/peritonitis/decreasing renal function, hemicolectomy
51	79	103	*Pseudomonas aeruginosa*	7	Postop repair anastomosis/peritonitis/decreasing renal function, hemicolectomy
51	79	98	*Pseudomonas aeruginosa*	1	Postop repair anastomosis/peritonitis/decreasing renal function, hemicolectomy
52	75	33	*Enterobacter cloacae*	1	Postop repair abdominal aortic aneurysm, renal failure, sepsis
52	75	34	*Enterobacter cloacae*	4	Postop repair abdominal aortic aneurysm, renal failure, sepsis
52	75	35	*Enterobacter cloacae*	6	Postop repair abdominal aortic aneurysm, renal failure, sepsis
53	81	144	*Pseudomonas aeruginosa*	1	Postop nephrectomy, pneumonia, resp failure, dementia
53	81	145	*Pseudomonas aeruginosa*	1	Postop nephrectomy, pneumonia, resp failure, dementia
54	26	149	*Enterobacter cloacae*	3	Septic shock, faecal peritonitis, perianal abscess, panproctocolectomy Crohn's disease
54	26	150	*Enterobacter cloacae*	12	Septic shock, faecal peritonitis, perianal abscess, panproctocolectomy Crohn's disease
55	19	198	*Pseudomonas aeruginosa*	1	Renal failure, septic shock, crush injuries, hemicolectomy perf colon, abd abscess, pancreatitis
56	61	4	*Citrobacter freundii*	1	Respiratory failure, cardiac failure, ARDS/ infection, COAD
56	61	5	*Citrobacter freundii*	3	Respiratory failure, cardiac failure, ARDS/ infection, COAD
57	45	86	*Acinetobacter calco var anitratus*	1	Postop re-exploration, splenectomy after oesophagectomy for adenoca
58	57	27	*Pseudomonas aeruginosa*	8	Chest infection, PTE, late-onset asthma

Appendix I : Patients, Isolates and Clinical Details

Patient's No	Age	Isolate No	Isolate	Day	Clinical Summary
59	61	91	*Pseudomonas aeruginosa*	2	Postop repair RIH, resp & renal failure, bowel obstruction, pneumonia, division of adhesions
59	61	92	*Enterobacter cloacae*	2	Postop repair RIH, resp & renal failure, bowel obstruction, pneumonia, division of adhesions
59	61	93	*Pseudomonas aeruginosa*	4	Postop repair RIH, resp & renal failure, bowel obstruction, pneumonia, division of adhesions
59	61	94	*Enterobacter cloacae*	4	Postop repair RIH, resp & renal failure, bowel obstruction, pneumonia, division of adhesions
60	59	39	*Enterobacter cloacae*	2	Resp and CV monitoring after CAVG x 2, and MV Annuloplasty
60	59	40	*Enterobacter cloacae*	2	Resp and CV monitoring after CAVG x 2, and MV Annuloplasty
61	74	116	*Enterobacter cloacae*	4	Postop bilat axillo-fem grafts, repair abd aortic aneurysm, MI, ARF,sepsis
61	74	117	*Pseudomonas aeruginosa*	15	Postop bilat axillo-fem grafts, repair abd aortic aneurysm, MI, ARF,sepsis
62	57	77	*Enterobacter cloacae*	1	Postop polyagastrectomy for HD monitoring
62	57	78	*Enterobacter cloacae*	1	Postop polyagastrectomy for HD monitoring
62	57	79	*Enterobacter cloacae*	2	Postop polyagastrectomy for HD monitoring
62	57	80	*Enterobacter cloacae*	2	Postop polyagastrectomy for HD monitoring
62	57	81	*Enterobacter cloacae*	2	Postop polyagastrectomy for HD monitoring
63	66	127	*Acinetobacter calco var anitratus*	1	Shock team transfer post oesophagectomy
64	73	42	*Pseudomonas aeruginosa*	4	Shock team transfer post cholecystectomy, renal failure
64	73	41	*Pseudomonas aeruginosa*	1	Shock team transfer post cholecystectomy, renal failure
66	59	138	*Citrobacter freundii*	3	Post resp arrest/resp failure/renal failure
67	62	121	*Enterobacter* species	5	Respiratory failure following CABG
67	62	120	*Acinetobacter calco var anitratus*	5	Respiratory failure following CABG
68	73	113	*Morganella morganii*	8	Shock team transfer acute exacbn of COAD
69	23	13	*Acinetobacter calco var anitratus*	2	Multiple trauma motorcycle accident
69	23	15	*Acinetobacter calco var anitratus*	10	Multiple trauma motorcycle accident
70	61	95	*Pseudomonas fluorescens*	1	Postop laparotomy for peritonitis, resp & renal failure
70	61	96	*Acinetobacter calco var anitratus*	1	Postop laparotomy for peritonitis, resp & renal failure
70	61	97	*Citrobacter freundii*	5	Postop laparotomy for peritonitis, resp & renal failure
71	73	199	*Enterobacter cloacae*	2	Postop drainage subphrenic abscess, prev repair of aortic aneurysm
72	61	104	*Pseudomonas species*	7	Resp arrest, hypoxic cerebral damage, L hemiparesis, acute exacbn COAD
73	70	89	*Flavobacterium odoratum*	4	Postop oesophagogastrectomy oesophageal ca
73	70	90	*Flavobacterium odoratum*	4	Postop oesophagogastrectomy oesophageal ca
74	77	170	*Pseudomonas aeruginosa*	2	Postop hemicolectomy, respiratory distress
74	77	172	*Pseudomonas aeruginosa*	4	Postop hemicolectomy, respiratory distress
75	57	47	*Enterobacter cloacae*	1	Necrotising fasciitis, excision of dead tissue from abdominal wall
76	56	22	*Enterobacter cloacae*	1	Postop resection small bowel, saphenous graft for SMA thrombosis
77	50	130	*Acinetobacter calco var anitratus*	1	Postop excision of aneurysm, tracheostomy, prolonged ventilation, NHL, sepsis.
78	21	162	*Pseudomonas aeruginosa*	2	Resp failure, cystic fibrosis

Appendix I : Patients, Isolates and Clinical Details

Patient's No	Age	Isolate No	Isolate	Day	Clinical Summary
80	67	154	*Acinetobacter calco var anitratus*	13	MI, CABG, perf DU, polyagastrectomy, septal rupture, cardiac arrest
80	67	151	*Pseudomonas aeruginosa*	1	MI, CABG, perf DU, polyagastrectomy, septal rupture, cardiac arrest
80	67	152	*Pseudomonas aeruginosa*	10	MI, CABG, perf DU, polyagastrectomy, septal rupture, cardiac arrest
81	29	140	*Klebsiella pneum pneumoniae*	11	Respiratory failure, Hodgkin's disease, prev bone marrow transplant
81	29	137	*Acinetobacter* species	8	Respiratory failure, Hodgkin's disease, prev bone marrow transplant
81	29	132	*Acinetobacter calco var anitratus*	6	Respiratory failure, Hodgkin's disease, prev bone marrow transplant
81	29	133	*Klebsiella pneum pneumoniae*	6	Respiratory failure, Hodgkin's disease, prev bone marrow transplant
82	59	23	*Morganella morganii*	1	Postop closure of ventral hernia, mitral valve disease, emergency desloughing abd wall
82	59	24	*Morganella morganii*	3	Postop closure of ventral hernia, mitral valve disease, emergency desloughing abd wall
82	59	25	*Morganella morganii*	1	Postop closure of ventral hernia, mitral valve disease, emergency desloughing abd wall
83	66	190	*Pseudomonas aeruginosa*	3	Renal failure after dissection & repair aortic aneurysm
84	50	88	*Acinetobacter calco var anitratus*	1	HD monitoring/COAD/chest infection
85	72	84	*Pseudomonas aeruginosa*	4	Cardioresp arrest, chest infection/LVF
85	72	83	*Pseudomonas aeruginosa*	6	Cardioresp arrest, chest infection/LVF
85	72	85	*Pseudomonas aeruginosa*	1	Cardioresp arrest, chest infection/LVF
86	51	75	*Hafnia alvei*	1	Resp failure, disseminated lymphoma, DIC, diabetes
87	65	118	*Citrobacter freundii*	1	Septic shock following biliary bypass surgery
87	65	119	*Citrobacter freundii*	4	Septic shock following biliary bypass surgery
87	65	122	*Citrobacter freundii*	6	Septic shock following biliary bypass surgery
88	63	52	*Enterobacter cloacae*	1	Sepsis, perf DU, total gastrectomy, subphrenic abscess
89	53	65	*Enterobacter cloacae*	14	Widespread necrotising fasciitis following CABG
89	53	66	*Enterobacter cloacae*	16	Widespread necrotising fasciitis following CABG
89	53	64	*Enterobacter cloacae*	11	Widespread necrotising fasciitis following CABG
89	53	62	*Enterobacter cloacae*	4	Widespread necrotising fasciitis following CABG
89	53	61	*Enterobacter cloacae*	2	Widespread necrotising fasciitis following CABG
90	75	163	*Pseudomonas aeruginosa*	5	Postop Hartman's procedure for perforated diverticulum
91	48	43	*Enterobacter cloacae*	12	Postop relief small bowel obstruction, division of adhesions, MS in remission
92	69	171	*Pseudomonas aeruginosa*	1	Postop choledochojejunostomy, obstructive jaundice, cholangiocarcinoma
93	44	7	*Enterobacter cloacae*	3	Postop TVR and 2nd MVR
93	44	8	*Enterobacter cloacae*	1	Postop TVR and 2nd MVR
94	64	21	*Pseudomonas aeruginosa*	4	Resp failure and pneumonia
95	59	45	*Stenotrophomonas maltophilia*	2	Postop pneumonectomy for infiltrating adenoca of left lung
95	59	46	*Acinetobacter calco var lwoffii*	2	Postop pneumonectomy for infiltrating adenoca of left lung
98	60	76	*Escherichia coli*	5	Postop pathological fracture femur, myeloma, bood loss
99	54	115	*Acinetobacter calco var anitratus*	1	Postop hemicolectomy/transplant nephrectomy

Appendix II : Isolates, Potential Recipients and their MICs

Isolate number	Isolate	Minimum inhibitory concentration (mg/L)		
		cefotaxime	rifampicin	nalidixic acid
2	*Acinetobacter calco var anitratus*	16	16	16
3	*Acinetobacter calco var anitratus*	16	31	4
4	*Citrobacter freundii*	16	31	8
5	*Citrobacter freundii*	16	62	8
6	*Acinetobacter calco var anitratus*	8	8	4
7	*Enterobacter cloacae*	>32	31	4
8	*Enterobacter cloacae*	>32	62	8
9	*Citrobacter freundii*	32	16	8
10	*Morganella morganii*	16	16	4
13	*Acinetobacter calco var anitratus*	32	8	16
15	*Acinetobacter calco var anitratus*	>32	16	32
16	*Morganella morganii*	32	16	4
17	*Pseudomonas aeruginosa*	32	62	>32
18	*Acinetobacter calco var lwoffii*	16	4	4
19	*Pseudomonas aeruginosa*	32	31	>32
20	*Pseudomonas aeruginosa*	32	31	>32
21	*Pseudomonas aeruginosa*	>32	62	>32
22	*Enterobacter cloacae*	32	31	8
23	*Morganella morganii*	>32	16	4
24	*Morganella morganii*	16	16	4
25	*Morganella morganii*	16	16	4
26	*Enterobacter cloacae*	32	31	16
27	*Pseudomonas aeruginosa*	>32	31	>32
28	*Stenotrophomonas maltophilia*	>32	16	16
29	*Stenotrophomonas maltophilia*	>32	16	16
30	*Stenotrophomonas maltophilia*	>32	16	16
31	*Stenotrophomonas maltophilia*	>32	16	16
32	*Stenotrophomonas maltophilia*	>32	16	16
33	*Enterobacter cloacae*	32	31	4
34	*Enterobacter cloacae*	32	31	4
35	*Enterobacter cloacae*	32	31	8
36	*Enterobacter cloacae*	>32	31	4
37	*Enterobacter cloacae*	>32	31	8
38	*Pseudomonas aeruginosa*	>32	62	>32
39	*Enterobacter cloacae*	>32	31	4
40	*Enterobacter cloacae*	>32	31	4
41	*Pseudomonas aeruginosa*	32	62	>32
42	*Pseudomonas aeruginosa*	32	62	>32
43	*Enterobacter cloacae*	32	31	4
45	*Stenotrophomonas maltophilia*	>32	16	8
46	*Acinetobacter calco var lwoffii*	32	4	8
47	*Enterobacter cloacae*	32	31	8
48	*Pseudomonas aeruginosa*	>32	62	>32
49	*Pseudomonas aeruginosa*	>32	62	>32
50	*Enterobacter cloacae*	32	62	8
51	*Enterobacter cloacae*	32	62	4
52	*Enterobacter cloacae*	>32	31	8
53	*Enterobacter cloacae*	>32	31	8
54	*Enterobacter cloacae*	>32	31	4
55	*Pseudomonas aeruginosa*	>32	62	>32
56	*Pseudomonas aeruginosa*	>32	62	>32
57	*Morganella morganii*	16	31	4
58	*Morganella morganii*	8	16	4
59	*Pseudomonas aeruginosa*	>32	62	>32
60	*Pseudomonas aeruginosa*	32	62	>32
61	*Enterobacter cloacae*	>32	31	8
62	*Enterobacter cloacae*	>32	31	8
63	*Pseudomonas aeruginosa*	>32	31	>32
64	*Enterobacter cloacae*	>32	31	16
65	*Enterobacter cloacae*	>32	31	8
66	*Enterobacter cloacae*	>32	31	8
67	*Pseudomonas* species	32	8	>32
68	*Pseudomonas* species	32	8	>32

Appendix II : Isolates, Potential Recipients and their MICs

Isolate number	Isolate	Minimum inhibitory concentration (mg/L)		
		cefotaxime	rifampicin	nalidixic acid
69	*Pseudomonas* species	32	8	>32
70	*Pseudomonas* species	16	8	>32
71	*Pseudomonas aeruginosa*	>32	31	>32
72	*Pseudomonas aeruginosa*	>32	31	>32
73	*Pseudomonas aeruginosa*	>32	31	>32
75	*Hafnia alvei*	32	62	4
76	*Escherichia coli*	4	16	4
77	*Enterobacter cloacae*	>32	31	8
78	*Enterobacter cloacae*	>32	31	8
79	*Enterobacter cloacae*	>32	62	8
80	*Enterobacter cloacae*	>32	31	8
81	*Enterobacter cloacae*	>32	31	8
82	*Acinetobacter calco var anitratus*	8	8	32
83	*Pseudomonas aeruginosa*	>32	62	>32
84	*Pseudomonas aeruginosa*	>32	31	>32
85	*Pseudomonas aeruginosa*	>32	31	>32
86	*Acinetobacter calco var anitratus*	32	8	16
87	*Pseudomonas aeruginosa*	>32	31	>32
88	*Acinetobacter calco var anitratus*	16	16	16
89	*Flavobacterium odoratum*	32	<2	8
90	*Flavobacterium odoratum*	32	4	8
91	*Pseudomonas aeruginosa*	>32	31	>32
92	*Enterobacter cloacae*	>32	31	4
93	*Pseudomonas aeruginosa*	>32	31	>32
94	*Enterobacter cloacae*	>32	31	8
95	*Pseudomonas fluorescens*	>32	31	>32
96	*Acinetobacter calco var anitratus*	32	8	16
97	*Citrobacter freundii*	>32	31	8
98	*Pseudomonas aeruginosa*	>32	31	>32
99	*Stenotrophomonas maltophilia*	>32	8	8
100	*Enterobacter cloacae*	>32	31	8
101	*Enterobacter cloacae*	>32	62	8
102	*Enterobacter cloacae*	>32	31	32
103	*Pseudomonas aeruginosa*	>32	31	>32
104	*Pseudomonas* species	>32	62	>32
105	*Acinetobacter calco var anitratus*	16	31	32
106	*Acinetobacter calco var anitratus*	16	8	32
107	*Acinetobacter calco var anitratus*	16	16	16
108	*Acinetobacter calco var anitratus*	16	16	>32
109	*Pseudomonas aeruginosa*	>32	62	>32
110	*Pseudomonas aeruginosa*	>32	31	>32
111	*Acinetobacter calco var anitratus*	32	8	16
112	*Acinetobacter calco var anitratus*	32	8	16
113	*Morganella morganii*	32	16	32
114	*Acinetobacter calco var anitratus*	32	62	16
115	*Acinetobacter calco var anitratus*	16	16	32
116	*Enterobacter cloacae*	>32	62	32
117	*Pseudomonas aeruginosa*	>32	31	>32
118	*Citrobacter freundii*	32	62	8
119	*Citrobacter freundii*	32	62	8
120	*Acinetobacter calco var anitratus*	32	62	16
121	*Enterobacter* species	>32	32	8
122	*Citrobacter freundii*	>32	32	8
123	*Acinetobacter calco var lwoffii*	16	8	8
124	*Pseudomonas aeruginosa*	>32	62	>32
125	*Pseudomonas aeruginosa*	>32	32	>32
126	*Enterobacter cloacae*	>32	31	16
127	*Acinetobacter calco var anitratus*	16	8	16
128	*Pseudomonas aeruginosa*	>32	16	>32
129	*Pseudomonas aeruginosa*	16	31	>32
130	*Acinetobacter calco var anitratus*	32	4	16
132	*Acinetobacter calco var anitratus*	16	8	16
133	*Klebsiella pneum pneumoniae*	<0.5	31	4

Appendix II : Isolates, Potential Recipients and their MICs

Isolate number	Isolate	Minimum inhibitory concentration (mg/L)		
		cefotaxime	rifampicin	nalidixic acid
134	*Enterobacter cloacae*	>32	31	8
135	*Enterobacter* species	>32	31	8
137	*Acinetobacter* species	16	8	8
138	*Citrobacter freundii*	8	8	4
139	*Pseudomonas aeruginosa*	>32	31	>32
140	*Klebsiella pneum pneumoniae*	<0.5	31	4
141	*Enterobacter cloacae*	>32	31	32
142	*Enterobacter cloacae*	>32	31	8
143	*Acinetobacter calco var anitratus*	8	8	8
144	*Pseudomonas aeruginosa*	>32	31	>32
145	*Pseudomonas aeruginosa*	>32	31	>32
146	*Enterobacter cloacae*	>32	31	>32
147	*Enterobacter cloacae*	>32	31	>32
148	*Enterobacter cloacae*	>32	31	>32
149	*Enterobacter cloacae*	>32	31	4
150	*Enterobacter cloacae*	>32	31	4
151	*Pseudomonas aeruginosa*	>32	31	>32
152	*Pseudomonas aeruginosa*	>32	31	>32
153	*Citrobacter freundii*	32	62	8
154	*Acinetobacter calco var anitratus*	16	8	16
157	*Morganella morganii*	16	16	4
158	*Pseudomonas aeruginosa*	>32	31	>32
160	*Pseudomonas aeruginosa*	>32	31	>32
161	*Enterobacter cloacae*	>32	31	>32
162	*Pseudomonas aeruginosa*	16	31	>32
163	*Pseudomonas aeruginosa*	>32	31	>32
165	*Citrobacter freundii*	>32	31	16
166	*Pseudomonas aeruginosa*	>32	31	>32
167	*Hafnia alvei*	>32	31	4
168	*Hafnia alvei*	>32	31	4
169	*Hafnia alvei*	32	62	4
170	*Pseudomonas aeruginosa*	32	31	>32
171	*Pseudomonas aeruginosa*	>32	31	>32
172	*Pseudomonas aeruginosa*	>32	31	>32
173	*Acinetobacter calco var anitratus*	>32	8	>32
174	*Enterobacter cloacae*	>32	31	16
177	*Pseudomonas aeruginosa*	>32	31	>32
178	*Enterobacter cloacae*	>32	16	16
182	*Pseudomonas aeruginosa*	32	62	>32
183	*Pseudomonas aeruginosa*	32	62	>32
184	*Enterobacter cloacae*	>32	16	8
190	*Pseudomonas aeruginosa*	32	31	>32
191	*Acinetobacter calco var anitratus*	16	8	16
195	*Acinetobacter calco var anitratus*	16	8	8
196	*Acinetobacter calco var anitratus*	16	8	16
197	*Enterobacter cloacae*	>32	31	8
198	*Pseudomonas aeruginosa*	>32	62	>32
199	*Enterobacter cloacae*	>32	62	>32
200	*Escherichia coli*	32	31	8
201	CT 73 *Escherichia coli*	N/A	8	N/A
202	J62-2 *Escherichia coli* SA 128	0.125	1000	N/A
203	DH5α *Escherichia coli*	<0.5	N/A	>32
			(N/A - Not applicable)	

Appendix III : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CN10	PRL100	AZL75	AMC30	FOX30	CIP5	IPM10	CAZ30	CAR100	TIC75	TIM85	AMP10	TZP110	ATM30
2	*Acinetobacter calco var anitratus*	19	24	15	25	0	30	34	25	23	24	30	0	29	13
3	*Acinetobacter calco var anitratus*	24	15	0	0	0	40	33	0	0	0	14	0	20	19
6	*Acinetobacter calco var anitratus*	15	20	15	24	15	27	30	25	25	25	25	0	25	20
13	*Acinetobacter calco var anitratus*	20	24	17	0	0	30	30	26	24	20	22	0	24	14
15	*Acinetobacter calco var anitratus*	22	19	0	15	0	25	36	22	17	19	22	0	22	0
82	*Acinetobacter calco var anitratus*	19	24	18	24	19	27	30	24	30	30	32	0	30	23
86	*Acinetobacter calco var anitratus*	26	19	15	20	15	24	34	26	18	16	24	11	23	16
88	*Acinetobacter calco var anitratus*	21	24	18	0	12	30	36	25	24	23	27	0	27	16
96	*Acinetobacter calco var anitratus*	23	23	19	25	10	29	32	25	25	25	30	0	28	17
105	*Acinetobacter calco var anitratus*	22	25	15	24	0	30	30	24	23	24	28	0	25	14
106	*Acinetobacter calco var anitratus*	0	24	23	26	0	24	34	22	25	24	30	0	23	17
107	*Acinetobacter calco var anitratus*	25	24	15	23	0	30	32	25	25	24	30	0	25	12
108	*Acinetobacter calco var anitratus*	24	24	15	27	0	30	36	22	22	20	32	0	26	0
111	*Acinetobacter calco var anitratus*	20	23	17	20	0	30	30	25	25	24	27	0	25	17
112	*Acinetobacter calco var anitratus*	20	24	16	19	0	32	32	26	25	25	25	0	25	17
114	*Acinetobacter calco var anitratus*	20	19	0	16	0	32	32	25	25	25	28	0	24	14
115	*Acinetobacter calco var anitratus*	20	25	19	25	0	30	32	24	23	24	29	0	27	0
120	*Acinetobacter calco var anitratus*	0	24	14	26	0	26	34	22	23	25	30	0	28	13
127	*Acinetobacter calco var anitratus*	22	25	15	25	0	34	32	25	24	22	29	0	27	15
130	*Acinetobacter calco var anitratus*	21	12	0	12	0	25	32	26	38	18	20	0	22	0
132	*Acinetobacter calco var anitratus*	0	28	19	26	0	22	34	26	26	27	30	0	36	17
143	*Acinetobacter calco var anitratus*	25	23	19	23	12	28	34	23	24	25	30	0	25	17
154	*Acinetobacter calco var anitratus*	28	28	19	24	0	32	34	36	32	30	34	0	34	18
173	*Acinetobacter calco var anitratus*	20	28	24	30	19	35	34	25	30	30	30	0	30	25
191	*Acinetobacter calco var anitratus*	20	25	17	24	0	30	36	25	25	25	30	13	25	20
195	*Acinetobacter calco var anitratus*	24	27	20	29	0	30	34	28	28	30	35	0	32	20
196	*Acinetobacter calco var anitratus*	22	24	14	24	0	30	36	25	25	25	36	0	30	12
18	*Acinetobacter calco var lwoffii*	35	22	0	0	0	36	25	22	0	0	26	0	25	0
46	*Acinetobacter calco var lwoffii*	21	18	0	0	0	30	25	0	0	0	13	0	19	0
123	*Acinetobacter calco var lwoffii*	24	25	0	24	0	28	38	28	28	24	30	0	20	0
137	*Acinetobacter* species	0	23	0	25	0	18	32	18	25	24	36	0	25	0
4	*Citrobacter freundii*	25	12	0	0	0	46	29	0	0	0	14	0	15	14
5	*Citrobacter freundii*	25	0	0	0	0	44	34	0	0	0	14	0	18	18
9	*Citrobacter freundii*	30	15	0	0	25	44	32	21	30	20	25	0	30	34
97	*Citrobacter freundii*	24	10	0	0	0	46	29	0	0	0	12	0	17	0
118	*Citrobacter freundii*	28	20	0	0	0	48	36	0	0	0	12	0	22	0
119	*Citrobacter freundii*	28	0	0	0	0	48	36	0	0	0	11	0	20	0

Appendix III : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CN10	PRL100	AZL75	AMC30	FOX30	CIP5	IPM10	CAZ30	CAR100	TIC75	TIM85	AMP10	TZP110	ATM30
122	*Citrobacter freundii*	30	14	0	0	0	40	34	0	0	0	0	0	20	30
138	*Citrobacter freundii*	24	0	0	0	0	40	30	0	0	0	0	0	29	0
153	*Citrobacter freundii*	30	20	0	0	0	40	30	0	0	0	0	0	25	0
165	*Citrobacter freundii*	25	0	0	0	0	34	30	0	0	0	0	0	17	13
7	*Enterobacter cloacae*	25	23	0	0	0	38	32	0	0	0	10	0	23	15
8	*Enterobacter cloacae*	24	25	0	0	0	40	36	0	0	0	13	0	24	17
22	*Enterobacter cloacae*	25	23	0	0	0	42	32	0	0	0	0	0	24	0
26	*Enterobacter cloacae*	25	20	0	0	0	40	32	15	0	0	13	0	22	15
33	*Enterobacter cloacae*	28	23	0	0	0	36	28	0	0	0	0	0	22	0
34	*Enterobacter cloacae*	25	22	0	0	0	38	30	12	0	0	0	0	24	16
35	*Enterobacter cloacae*	26	23	0	0	0	40	32	14	0	0	0	0	27	16
36	*Enterobacter cloacae*	36	17	0	0	0	36	32	0	14	0	15	0	23	21
37	*Enterobacter cloacae*	23	17	0	0	0	38	32	17	14	0	15	0	22	22
39	*Enterobacter cloacae*	25	22	0	0	0	42	32	14	0	0	14	0	23	22
40	*Enterobacter cloacae*	25	19	0	0	0	38	30	15	0	0	0	0	20	0
43	*Enterobacter cloacae*	24	22	0	0	0	44	28	17	11	0	10	0	22	19
47	*Enterobacter cloacae*	24	21	0	0	0	34	31	17	0	0	11	0	21	17
50	*Enterobacter cloacae*	20	18	0	0	15	40	30	17	17	15	18	0	21	19
51	*Enterobacter cloacae*	20	18	0	0	15	40	30	17	17	15	18	0	21	19
52	*Enterobacter cloacae*	23	0	0	0	0	34	27	0	0	0	12	0	15	0
53	*Enterobacter cloacae*	23	0	0	0	0	40	28	0	0	0	0	0	14	0
54	*Enterobacter cloacae*	22	0	0	0	0	36	24	0	0	0	0	0	12	0
61	*Enterobacter cloacae*	24	0	0	0	0	40	25	0	0	0	0	0	11	10
62	*Enterobacter cloacae*	23	0	0	0	0	38	24	0	0	0	10	0	11	9
64	*Enterobacter cloacae*	22	0	0	0	0	34	26	0	0	0	11	0	13	0
65	*Enterobacter cloacae*	22	0	0	0	0	40	26	0	0	0	0	0	15	13
66	*Enterobacter cloacae*	20	0	0	0	0	30	29	0	0	0	0	0	12	0
77	*Enterobacter cloacae*	0	25	12	0	0	38	28	28	26	14	14	0	28	28
78	*Enterobacter cloacae*	0	0	0	0	0	40	26	12	0	0	12	0	19	15
79	*Enterobacter cloacae*	27	25	0	0	0	34	27	28	14	0	10	0	25	30
80	*Enterobacter cloacae*	0	13	0	0	0	34	29	0	15	10	12	0	38	16
81	*Enterobacter cloacae*	0	20	12	0	0	40	28	30	28	0	14	0	30	32
92	*Enterobacter cloacae*	26	0	0	0	0	36	24	0	12	0	12	0	14	12
94	*Enterobacter cloacae*	22	0	0	0	0	22	30	0	0	0	0	0	15	14
100	*Enterobacter cloacae*	25	15	0	0	0	40	25	0	0	0	0	0	15	0
101	*Enterobacter cloacae*	29	20	0	0	0	40	26	0	0	0	0	0	16	0
102	*Enterobacter cloacae*	25	18	0	0	0	40	30	0	0	0	0	0	16	0

Appendix III : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CN10	PRL100	AZL75	AMC30	FOX30	CIP5	IPM10	CAZ30	CAR100	TIC75	TIM85	AMP10	TZP110	ATM30
116	*Enterobacter cloacae*	0	29	14	0	0	46	30	24	20	20	0	0	25	36
126	*Enterobacter cloacae*	25	19	0	0	0	38	32	0	0	0	13	0	20	0
134	*Enterobacter cloacae*	0	0	0	0	0	40	36	0	0	0	0	0	14	11
141	*Enterobacter cloacae*	0	0	0	0	0	40	30	0	0	0	12	0	15	11
142	*Enterobacter cloacae*	0	0	0	0	0	40	32	0	0	0	0	0	15	12
146	*Enterobacter cloacae*	25	0	0	0	0	25	36	0	0	0	12	0	20	0
147	*Enterobacter cloacae*	26	18	0	0	0	30	30	0	0	0	0	0	20	20
148	*Enterobacter cloacae*	28	0	0	0	0	28	35	0	0	0	0	0	19	0
149	*Enterobacter cloacae*	35	20	0	0	0	40	30	0	0	0	0	0	20	12
150	*Enterobacter cloacae*	24	15	0	0	0	44	25	0	0	0	0	0	14	0
161	*Enterobacter cloacae*	20	0	0	0	0	40	28	0	0	0	10	0	15	12
174	*Enterobacter cloacae*	19	20	0	0	0	40	30	18	0	0	12	0	20	20
178	*Enterobacter cloacae*	22	0	0	0	0	40	28	20	0	0	12	0	22	20
184	*Enterobacter cloacae*	20	25	0	0	0	35	30	20	0	0	12	0	25	24
197	*Enterobacter cloacae*	24	20	0	0	0	42	28	14	10	9	12	0	22	16
199	*Enterobacter cloacae*	22	20	0	0	0	30	27	18	16	12	14	0	20	19
121	*Enterobacter* species	0	28	0	0	0	44	30	30	0	0	0	0	30	30
135	*Enterobacter* species	0	0	0	0	0	40	30	0	0	0	12	0	15	12
76	*Escherichia coli*	22	0	0	0	28	36	30	30	0	0	14	0	13	34
200	*Escherichia coli*	24	18	0	0	13	32	30	21	0	0	15	0	18	20
89	*Flavobacterium odoratum*	0	25	20	0	20	30	0	21	0	9	12	0	20	0
90	*Flavobacterium odoratum*	0	28	19	0	22	30	0	22	0	7	12	0	25	0
75	*Hafnia alvei*	24	0	0	0	20	34	27	11	15	11	14	0	15	21
167	*Hafnia alvei*	30	20	0	0	30	42	30	0	17	18	22	0	19	25
168	*Hafnia alvei*	25	15	0	0	36	38	30	0	15	10	21	0	20	22
169	*Hafnia alvei*	30	20	0	0	30	42	35	16	0	19	28	0	20	30
133	*Klebsiella pneum pneumoniae*	0	11	0	11	27	34	32	20	0	0	0	0	28	30
140	*Klebsiella pneum pneumoniae*	0	12	0	11	28	34	30	16	0	0	0	0	27	40
10	*Morganella morganii*	30	14	0	0	24	44	32	20	30	20	25	0	29	34
16	*Morganella morganii*	24	21	0	0	20	44	24	20	20	20	23	0	28	34
23	*Morganella morganii*	22	11	0	0	0	46	24	0	0	0	0	0	0	0
24	*Morganella morganii*	24	17	0	0	22	44	27	19	20	21	25	0	28	30
25	*Morganella morganii*	23	15	0	0	22	44	30	17	25	20	23	0	25	28
57	*Morganella morganii*	25	16	0	0	0	40	26	12	18	0	0	0	20	22
58	*Morganella morganii*	24	24	0	0	15	44	25	30	30	21	24	0	36	38
113	*Morganella morganii*	27	15	0	0	24	48	30	19	30	21	24	0	26	32
157	*Morganella morganii*	28	20	0	0	27	35	27	25	36	26	30	0	30	36

Appendix III : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CN10	PRL100	AZL75	AMC30	FOX30	CIP5	IPM10	CAZ30	CAR100	TIC75	TIM85	AMP10	TZP110	ATM30
17	*Pseudomonas aeruginosa*	21	34	30	0	0	35	26	30	0	18	24	0	30	28
19	*Pseudomonas aeruginosa*	26	36	30	0	0	40	24	30	14	20	24	0	30	27
20	*Pseudomonas aeruginosa*	28	34	32	0	0	42	26	30	14	20	24	0	30	28
21	*Pseudomonas aeruginosa*	20	36	30	0	0	36	30	30	15	20	24	0	32	26
27	*Pseudomonas aeruginosa*	16	0	0	0	0	44	20	0	0	0	0	0	0	0
38	*Pseudomonas aeruginosa*	19	32	28	0	0	38	25	30	13	20	24	0	32	25
41	*Pseudomonas aeruginosa*	15	30	29	0	0	38	24	30	15	25	26	0	30	24
42	*Pseudomonas aeruginosa*	17	32	30	0	0	34	24	30	18	23	25	0	28	25
48	*Pseudomonas aeruginosa*	17	18	10	0	0	32	30	20	15	16	17	0	21	14
49	*Pseudomonas aeruginosa*	15	16	0	0	0	39	20	12	0	0	0	0	18	0
55	*Pseudomonas aeruginosa*	20	22	0	0	0	30	30	30	0	0	14	0	25	18
56	*Pseudomonas aeruginosa*	24	25	24	0	0	30	0	29	0	0	10	0	25	15
59	*Pseudomonas aeruginosa*	20	32	28	0	15	40	25	32	18	22	28	0	30	36
60	*Pseudomonas aeruginosa*	20	30	27	0	0	38	24	30	16	21	23	0	28	25
63	*Pseudomonas aeruginosa*	26	28	24	0	0	34	22	30	12	17	20	0	27	23
71	*Pseudomonas aeruginosa*	13	25	24	0	0	30	24	24	13	22	25	0	28	22
72	*Pseudomonas aeruginosa*	19	29	27	0	0	34	20	25	16	20	24	0	25	24
73	*Pseudomonas aeruginosa*	20	31	27	0	0	38	23	30	15	21	25	0	27	24
83	*Pseudomonas aeruginosa*	15	28	23	0	0	30	21	28	16	20	22	0	22	25
84	*Pseudomonas aeruginosa*	16	30	25	0	0	32	20	28	15	18	20	0	29	24
85	*Pseudomonas aeruginosa*	18	30	28	0	0	32	21	28	15	20	22	0	29	25
87	*Pseudomonas aeruginosa*	19	30	28	0	0	32	26	27	19	22	24	0	30	24
91	*Pseudomonas aeruginosa*	16	30	30	0	0	32	29	30	19	21	24	0	30	30
93	*Pseudomonas aeruginosa*	19	17	0	0	0	38	28	20	0	12	15	0	18	19
98	*Pseudomonas aeruginosa*	22	34	32	0	0	40	27	34	22	24	27	0	36	30
103	*Pseudomonas aeruginosa*	22	20	12	0	0	40	28	18	13	0	14	0	20	14
109	*Pseudomonas aeruginosa*	20	38	32	0	0	40	32	32	18	24	25	0	32	29
110	*Pseudomonas aeruginosa*	20	34	32	0	0	42	25	34	18	27	30	0	34	30
117	*Pseudomonas aeruginosa*	22	0	0	0	0	40	22	0	0	0	12	0	0	0
124	*Pseudomonas aeruginosa*	24	25	15	27	0	32	36	29	26	28	32	0	30	14
125	*Pseudomonas aeruginosa*	20	30	27	0	0	32	28	28	18	23	24	0	30	24
129	*Pseudomonas aeruginosa*	20	34	30	0	0	40	22	34	20	24	25	0	30	29
139	*Pseudomonas aeruginosa*	19	25	24	0	0	32	21	28	13	18	20	0	25	22
144	*Pseudomonas aeruginosa*	20	32	27	0	0	28	22	30	15	25	24	0	30	29
145	*Pseudomonas aeruginosa*	20	32	30	0	0	34	22	30	18	23	24	0	30	22
151	*Pseudomonas aeruginosa*	20	30	28	0	0	44	25	30	20	24	25	0	30	30
152	*Pseudomonas aeruginosa*	20	36	25	0	0	38	28	28	15	20	21	0	27	28

Appendix III : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CN10	PRL100	AZL75	AMC30	FOX30	CIP5	IPM10	CAZ30	CAR100	TIC75	TIM85	AMP10	TZP110	ATM30
160	*Pseudomonas aeruginosa*	20	36	30	0	0	20	25	28	18	26	30	0	36	24
162	*Pseudomonas aeruginosa*	17	32	30	0	0	30	20	30	15	24	25	0	30	27
163	*Pseudomonas aeruginosa*	19	25	25	0	0	17	29	25	12	20	24	0	28	25
166	*Pseudomonas aeruginosa*	17	34	30	0	0	36	25	30	17	20	25	0	30	25
170	*Pseudomonas aeruginosa*	15	28	25	0	0	30	25	30	20	25	25	0	30	36
171	*Pseudomonas aeruginosa*	18	26	14	0	0	38	21	21	20	17	17	0	24	24
172	*Pseudomonas aeruginosa*	18	30	27	0	0	34	28	30	18	24	25	0	30	27
177	*Pseudomonas aeruginosa*	22	30	28	0	0	44	25	30	20	25	25	0	36	30
182	*Pseudomonas aeruginosa*	20	35	30	0	0	40	22	30	15	22	25	0	30	30
183	*Pseudomonas aeruginosa*	19	35	30	0	0	40	22	27	15	20	25	0	36	25
190	*Pseudomonas aeruginosa*	18	30	30	0	0	36	24	30	16	21	25	0	30	28
198	*Pseudomonas aeruginosa*	12	28	26	0	0	30	19	30	16	23	24	0	34	28
95	*Pseudomonas fluorescens*	25	30	29	0	0	35	24	24	0	0	0	0	34	0
67	*Pseudomonas* species	34	34	28	23	0	40	44	34	14	15	24	0	34	18
68	*Pseudomonas* species	34	35	34	22	0	44	40	30	14	17	20	0	34	20
69	*Pseudomonas* species	32	30	30	20	0	44	36	36	10	10	20	0	34	16
70	*Pseudomonas* species	30	30	30	20	0	38	44	28	10	12	22	0	32	10
104	*Pseudomonas* species	20	27	22	0	0	30	26	30	18	21	24	0	32	25
128	*Pseudomonas* species	20	32	28	0	0	38	22	18	18	25	25	0	30	29
158	*Pseudomonas* species	18	30	28	0	0	35	20	30	17	24	24	0	30	25
28	*Stenotrophomonas maltophilia*	0	0	0	0	0	22	R	0	0	0	30	0	0	0
29	*Stenotrophomonas maltophilia*	0	0	0	0	0	17	0	0	0	0	16	0	0	0
30	*Stenotrophomonas maltophilia*	0	0	0	0	0	22	0	0	0	0	35	0	0	0
31	*Stenotrophomonas maltophilia*	0	0	0	0	0	25	0	0	0	0	35	0	0	0
32	*Stenotrophomonas maltophilia*	0	0	0	0	0	25	0	0	0	0	19	0	0	0
45	*Stenotrophomonas maltophilia*	0	22	26	0	0	30	0	28	20	18	44	0	30	0
99	*Stenotrophomonas maltophilia*	0	0	0	0	0	22	0	0	0	0	25	0	0	0

Appendix IV : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CTX30	MEM10	MEL25	TEM30	CTX MIC	ESBL_TZ	ESBL_TZL	ESBL: POS, NEG or ND
2	*Acinetobacter calco var anitratus*	19	30	0	0	16	6	>8	N
3	*Acinetobacter calco var anitratus*	14	35	36	20	16	>32	>8	ND
6	*Acinetobacter calco var anitratus*	20	27	24	0	8	>32	>8	ND
13	*Acinetobacter calco var anitratus*	25	30	0	0	32	12	>8	N
15	*Acinetobacter calco var anitratus*	0	28	0	0	>32	>32	>8	ND
82	*Acinetobacter calco var anitratus*	22	27	0	0	8	2	>8	N
86	*Acinetobacter calco var anitratus*	19	28	0	0	32	6	>8	N
88	*Acinetobacter calco var anitratus*	22	30	12	0	16	8	>8	N
96	*Acinetobacter calco var anitratus*	22	25	14	0	32	12	>8	N
105	*Acinetobacter calco var anitratus*	22	28	14	0	16	16	>8	N
106	*Acinetobacter calco var anitratus*	20	25	15	0	16	>32	>8	ND
107	*Acinetobacter calco var anitratus*	24	30	12	0	16	8	>8	N
108	*Acinetobacter calco var anitratus*	18	30	0	0	16	>32	>8	ND
111	*Acinetobacter calco var anitratus*	22	27	12	0	32	6	>8	N
112	*Acinetobacter calco var anitratus*	22	36	12	0	32	6	>8	N
114	*Acinetobacter calco var anitratus*	20	30	0	0	32	12	>8	N
115	*Acinetobacter calco var anitratus*	20	27	12	0	16	>32	>8	ND
120	*Acinetobacter calco var anitratus*	20	28	30	0	32	>32	>8	ND
127	*Acinetobacter calco var anitratus*	21	32	15	0	16	12	>8	N
130	*Acinetobacter calco var anitratus*	15	27	0	0	32	>32	>8	ND
132	*Acinetobacter calco var anitratus*	22	30	15	0	16	>32	0.38	P
143	*Acinetobacter calco var anitratus*	22	26	15	0	8	>32	>8	ND
154	*Acinetobacter calco var anitratus*	24	34	22	0	16	<0.5	<0.125	ND
173	*Acinetobacter calco var anitratus*	25	30	17	16	>32	2	>8	N
191	*Acinetobacter calco var anitratus*	25	30	12	0	16	0.12	>8	N
195	*Acinetobacter calco var anitratus*	26	32	19	0	16	>32	>8	ND
196	*Acinetobacter calco var anitratus*	20	30	12	0	16	>32	>8	ND
18	*Acinetobacter calco var lwoffii*	20	24	0	0	16	>32	>8	ND
46	*Acinetobacter calco var lwoffii*	0	30	32	0	32	>32	>8	ND
123	*Acinetobacter calco var lwoffii*	20	34	0	0	16	>32	>8	ND
137	*Acinetobacter* species	18	25	0	0	16	>32	<0.125	P
4	*Citrobacter freundii*	12	26	34	20	16	>32	>8	ND
5	*Citrobacter freundii*	15	38	40	15	16	>32	>8	ND
9	*Citrobacter freundii*	22	25	34	30	32	>32	>8	ND
97	*Citrobacter freundii*	0	34	32	13	>32	>32	>8	ND
118	*Citrobacter freundii*	0	38	40	0	32	>32	>8	ND
119	*Citrobacter freundii*	0	38	40	18	32	>32	>8	ND

Appendix IV : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CTX30	MEM10	MEL25	TEM30	CTX MIC	ESBL_TZ	ESBL_TZL	ESBL: POS, NEG or ND
122	*Citrobacter freundii*	0	36	40	12	>32	>32	>8	ND
138	*Citrobacter freundii*	0	36	38	0	8	>32	>8	ND
153	*Citrobacter freundii*	20	36	40	18	32	>32	>8	ND
165	*Citrobacter freundii*	0	37	36	14	>32	>32	>8	ND
7	*Enterobacter cloacae*	10	36	32	14	>32	>32	>8	ND
8	*Enterobacter cloacae*	18	38	34	17	>32	>32	>8	ND
22	*Enterobacter cloacae*	0	34	38	15	32	>32	>8	ND
26	*Enterobacter cloacae*	16	34	32	17	32	>32	>8	ND
33	*Enterobacter cloacae*	0	36	30	0	32	>32	>8	ND
34	*Enterobacter cloacae*	0	32	32	16	32	>32	>8	ND
35	*Enterobacter cloacae*	0	32	36	16	32	>32	>8	ND
36	*Enterobacter cloacae*	12	38	36	24	>32	>32	>8	ND
37	*Enterobacter cloacae*	12	34	34	21	>32	>32	>8	ND
39	*Enterobacter cloacae*	0	34	38	21	>32	>32	>8	ND
40	*Enterobacter cloacae*	0	34	34	0	>32	>32	>8	ND
43	*Enterobacter cloacae*	12	32	34	18	32	>32	>8	ND
47	*Enterobacter cloacae*	16	32	30	13	32	>32	>8	ND
50	*Enterobacter cloacae*	12	31	30	19	32	>32	>8	ND
51	*Enterobacter cloacae*	12	31	30	19	32	>32	>8	ND
52	*Enterobacter cloacae*	0	32	32	0	>32	>32	>8	ND
53	*Enterobacter cloacae*	0	26	30	0	>32	>32	>8	ND
54	*Enterobacter cloacae*	0	26	24	0	>32	>32	>8	ND
61	*Enterobacter cloacae*	0	27	24	0	>32	>32	>8	ND
62	*Enterobacter cloacae*	0	25	22	0	>32	>32	>8	ND
64	*Enterobacter cloacae*	0	28	25	12	>32	>32	>8	ND
65	*Enterobacter cloacae*	0	30	27	15	>32	>32	>8	ND
66	*Enterobacter cloacae*	0	28	26	12	>32	>32	>8	ND
77	*Enterobacter cloacae*	25	34	22	22	>32	2	>8	N
78	*Enterobacter cloacae*	0	28	24	14	>32	>32	>8	ND
79	*Enterobacter cloacae*	27	32	30	20	>32	2	>8	N
80	*Enterobacter cloacae*	0	30	30	17	>32	>32	>8	ND
81	*Enterobacter cloacae*	20	31	24	0	>32	3	>8	N
92	*Enterobacter cloacae*	0	27	24	12	>32	>32	>8	ND
94	*Enterobacter cloacae*	0	32	28	0	>32	>32	>8	ND
100	*Enterobacter cloacae*	0	30	26	0	>32	>32	>8	ND
101	*Enterobacter cloacae*	0	27	27	0	>32	>32	>8	ND
102	*Enterobacter cloacae*	0	30	30	0	>32	>32	>8	ND

Appendix IV : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CTX30	MEM10	MEL25	TEM30	CTX MIC	ESBL_TZ	ESBL_TZL	ESBL: POS, NEG or ND
116	*Enterobacter cloacae*	25	36	28	18	>32	3	>8	N
126	*Enterobacter cloacae*	0	34	38	15	>32	>32	>8	ND
134	*Enterobacter cloacae*	0	32	30	0	>32	>32	>8	ND
141	*Enterobacter cloacae*	0	30	29	14	>32	>32	>8	ND
142	*Enterobacter cloacae*	0	36	30	12	>32	>32	>8	ND
146	*Enterobacter cloacae*	0	38	38	20	>32	>32	>8	ND
147	*Enterobacter cloacae*	0	30	35	18	>32	>32	>8	ND
148	*Enterobacter cloacae*	0	35	40	17	>32	>32	>8	ND
149	*Enterobacter cloacae*	0	30	32	13	>32	>32	>8	ND
150	*Enterobacter cloacae*	0	30	30	0	>32	>32	>8	ND
161	*Enterobacter cloacae*	0	30	28	12	>32	>32	>8	ND
174	*Enterobacter cloacae*	12	38	35	20	>32	>32	>8	ND
178	*Enterobacter cloacae*	17	30	0	0	>32	>32	>8	ND
184	*Enterobacter cloacae*	17	35	30	20	>32	>32	>8	ND
197	*Enterobacter cloacae*	15	31	30	17	>32	>32	>8	ND
199	*Enterobacter cloacae*	20	31	28	16	>32	>32	>8	ND
121	*Enterobacter species*	30	34	30	22	>32	4	>8	N
135	*Enterobacter species*	0	36	28	13	>32	>32	>8	ND
76	*Escherichia coli*	28	34	0	20	4	<0.5	0.19	N
200	*Escherichia coli*	21	38	34	10	32	>32	>8	ND
89	*Flavobacterium odoratum*	12	0	0	0	32	>32	>8	ND
90	*Flavobacterium odoratum*	12	0	0	0	32	>32	>8	ND
75	*Hafnia alvei*	12	30	30	0	32	>32	>8	ND
167	*Hafnia alvei*	20	36	40	36	>32	>32	>8	ND
168	*Hafnia alvei*	17	40	44	14	>32	>32	>8	ND
169	*Hafnia alvei*	20	42	42	20	32	>32	>8	ND
133	*Klebsiella pneum pneumoniae*	38	34	22	25	<0.5	8	0.25	P
140	*Klebsiella pneum pneumoniae*	36	30	20	17	<0.5	>32	0.75	P
10	*Morganella morganii*	22	42	0	25	16	>32	>8	ND
16	*Morganella morganii*	22	36	0	24	32	> 32	>8	ND
23	*Morganella morganii*	0	32	0	14	>32	>32	>8	ND
24	*Morganella morganii*	22	38	0	25	16	>32	>8	ND
25	*Morganella morganii*	24	36	25	25	16	>32	>8	ND
57	*Morganella morganii*	15	38	0	18	16	>32	>8	ND
58	*Morganella morganii*	30	36	0	25	8	>32	>8	ND
113	*Morganella morganii*	22	38	0	25	32	>32	>8	ND
157	*Morganella morganii*	25	46	0	25	16	>32	>8	ND

Appendix IV : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CTX30	MEM10	MEL25	TEM30	CTX MIC	ESBL_TZ	ESBL_TZL	ESBL: POS, NEG or ND
17	*Pseudomonas aeruginosa*	18	36	0	0	32	>32	>8	ND
19	*Pseudomonas aeruginosa*	20	30	0	0	32	>32	>8	ND
20	*Pseudomonas aeruginosa*	22	30	0	0	32	>32	>8	ND
21	*Pseudomonas aeruginosa*	17	38	0	0	>32	4	>8	N
27	*Pseudomonas aeruginosa*	0	30	0	0	>32	>32	>8	ND
38	*Pseudomonas aeruginosa*	12	32	0	0	>32	2	8	N
41	*Pseudomonas aeruginosa*	18	34	0	0	32	8	>8	N
42	*Pseudomonas aeruginosa*	18	30	0	0	32	>32	>8	ND
48	*Pseudomonas aeruginosa*	0	32	0	0	>32	>32	>8	ND
49	*Pseudomonas aeruginosa*	0	29	0	0	>32	>32	>8	ND
55	*Pseudomonas aeruginosa*	0	30	0	0	>32	4	>8	N
56	*Pseudomonas aeruginosa*	0	0	0	0	>32	8	>8	N
59	*Pseudomonas aeruginosa*	19	34	0	0	>32	6	>8	N
60	*Pseudomonas aeruginosa*	16	30	0	0	32	6	>8	N
63	*Pseudomonas aeruginosa*	15	30	0	0	>32	4	>8	N
71	*Pseudomonas aeruginosa*	14	24	0	0	>32	4	>8	N
72	*Pseudomonas aeruginosa*	20	31	0	0	>32	2	>8	N
73	*Pseudomonas aeruginosa*	20	38	0	0	>32	2	6	N
83	*Pseudomonas aeruginosa*	19	28	0	0	>32	3	>8	N
84	*Pseudomonas aeruginosa*	18	27	0	0	>32	3	>8	N
85	*Pseudomonas aeruginosa*	18	28	0	0	>32	4	>8	N
87	*Pseudomonas aeruginosa*	18	36	0	0	>32	4	>8	N
91	*Pseudomonas aeruginosa*	22	30	0	0	>32	4	>8	N
93	*Pseudomonas aeruginosa*	0	29	0	0	>32	6	>8	N
98	*Pseudomonas aeruginosa*	23	38	0	0	>32	1	2	N
103	*Pseudomonas aeruginosa*	0	36	0	0	>32	>32	>8	ND
109	*Pseudomonas aeruginosa*	20	36	0	0	>32	8	>8	N
110	*Pseudomonas aeruginosa*	22	36	0	0	>32	3	>8	N
117	*Pseudomonas aeruginosa*	0	22	0	0	>32	>32	>8	ND
124	*Pseudomonas aeruginosa*	24	40	16	0	>32	12	>8	N
125	*Pseudomonas aeruginosa*	20	36	0	0	>32	3	>8	N
129	*Pseudomonas aeruginosa*	20	28	0	0	16	2	>8	N
139	*Pseudomonas aeruginosa*	16	36	0	0	>32	4	>8	N
144	*Pseudomonas aeruginosa*	19	30	0	0	>32	4	>8	N
145	*Pseudomonas aeruginosa*	22	28	0	0	>32	4	>8	N
151	*Pseudomonas aeruginosa*	20	35	0	0	>32	>32	>8	ND
152	*Pseudomonas aeruginosa*	20	33	0	0	>32	4	>8	N

Appendix IV : Isolates - Antibiotic Sensitivity

Isolate No	Isolate	CTX30	MEM10	MEL25	TEM30	CTX MIC	ESBL_TZ	ESBL_TZL	ESBL: POS, NEG or ND
160	*Pseudomonas aeruginosa*	20	42	0	0	>32	2	>8	N
162	*Pseudomonas aeruginosa*	20	28	0	0	16	2	>8	N
163	*Pseudomonas aeruginosa*	17	34	0	0	>32	4	>8	N
166	*Pseudomonas aeruginosa*	17	30	0	0	>32	3	6	N
170	*Pseudomonas aeruginosa*	17	34	0	0	32	2	8	N
171	*Pseudomonas aeruginosa*	0	38	0	0	>32	>32	>8	ND
172	*Pseudomonas aeruginosa*	20	34	0	0	>32	2	4	N
177	*Pseudomonas aeruginosa*	20	36	0	0	>32	2	3	N
182	*Pseudomonas aeruginosa*	22	30	0	0	32	>32	>8	ND
183	*Pseudomonas aeruginosa*	21	30	0	0	32	3	>8	N
190	*Pseudomonas aeruginosa*	20	34	0	0	32	3	>8	N
198	*Pseudomonas aeruginosa*	17	28	0	0	>32	4	>8	N
95	*Pseudomonas fluorescens*	0	30	0	0	>32	6	>8	N
67	*Pseudomonas* species	23	48	0	0	32	3	>8	N
68	*Pseudomonas* species	24	40	10	0	32	3	>8	N
69	*Pseudomonas* species	25	44	0	0	32	4	>8	N
70	*Pseudomonas* species	24	34	0	0	16	6	>8	N
104	*Pseudomonas* species	15	38	0	0	>32	4	>8	N
128	*Pseudomonas* species	21	27	0	0	>32	1.5	>8	N
158	*Pseudomonas* species	17	30	0	0	>32	3	>8	N
28	*Stenotrophomonas maltophilia*	0	0	0	0	>32	>32	>8	ND
29	*Stenotrophomonas maltophilia*	0	0	0	0	>32	>32	>8	ND
30	*Stenotrophomonas maltophilia*	0	0	0	0	>32	>32	>8	ND
31	*Stenotrophomonas maltophilia*	0	0	0	0	>32	>32	>8	ND
32	*Stenotrophomonas maltophilia*	0	0	0	0	>32	>32	>8	ND
45	*Stenotrophomonas maltophilia*	0	0	0	0	>32	>32	>8	ND
99	*Stenotrophomonas maltophilia*	0	0	0	0	>32	>32	>8	ND

Appendix V : Isolate, Plasmid and B-Lactamase Matrix

Isolate		Plasmids (kb)									B - Lactamases (pI)				
No.	Name	1	2	3	4	5	6	7	8	9	1	2	3	4	5
2	*Acinetobacter calco* var *anitratus*										8.5				
3	*Acinetobacter calco* var *anitratus*	8.27									8.3				
4	*Citrobacter freundii*										8.3				
5	*Citrobacter freundii*										8.3				
6	*Acinetobacter calco* var *anitratus*	142.1									8.3				
7	*Enterobacter cloacae*	93.47									8.3				
8	*Enterobacter cloacae*	93.47									8.3				
9	*Citrobacter freundii*	125.4									8.3				
10	*Morganella morganii*	71.25									7.4				
13	*Acinetobacter calco* var *anitratus*	12.83									7.8				
15	*Acinetobacter calco* var *anitratus*	11.71													
16	*Morganella morganii*										7.9				
17	*Pseudomonas aeruginosa*														
18	*Acinetobacter calco* var *lwoffii*	2.68	3.48	4.2	5.45	6.1	9.45	14.82	28.54	142.1	7.8				
19	*Pseudomonas aeruginosa*										8.2				
20	*Pseudomonas aeruginosa*										8.1				
21	*Pseudomonas aeruginosa*										7.8				
22	*Enterobacter cloacae*	4.84	10.11								7.9				
23	*Morganella morganii*	5.86	11.71	47.21							6.5	7.8			
24	*Morganella morganii*	5.86	11.71	47.21							7.7				
25	*Morganella morganii*	5.78	14.59								6.5	7.9			
26	*Enterobacter cloacae*	4.34	124.7								7.7				
27	*Pseudomonas aeruginosa*										7.9				
28	*Stenotrophomonas maltophilia*										6.1				
29	*Stenotrophomonas maltophilia*										6.1				
30	*Stenotrophomonas maltophilia*										6.1				
31	*Stenotrophomonas maltophilia*										6.1				
32	*Stenotrophomonas maltophilia*										6.1				
33	*Enterobacter cloacae*										8.1	8.7			
34	*Enterobacter cloacae*										8.1	8.7			
35	*Enterobacter cloacae*										8.0	8.4			
36	*Enterobacter cloacae*	124.7									8.1				
37	*Enterobacter cloacae*	124.7									8.1				

Appendix V : Isolate, Plasmid and B-Lactamase Matrix

Isolate		Plasmids (kb)									B - Lactamases (pI)				
No.	Name	1	2	3	4	5	6	7	8	9	1	2	3	4	5
38	*Pseudomonas aeruginosa*	43.45									7.9	8.8			
39	*Enterobacter cloacae*	5.67	11.32	18.36	30.98	53	124.7				8.0	8.8			
40	*Enterobacter cloacae*	5.67	18.36	30.98	53	101.9	124.7				8.3				
41	*Pseudomonas aeruginosa*														
42	*Pseudomonas aeruginosa*														
43	*Enterobacter cloacae*	5.15	77.21								5.6	5.8	6.2	7.7	8.8
45	*Stenotrophomonas maltophilia*	3.97	7.58	11.87											
46	*Acinetobacter calco* var *lwoffii*	8.83	97.15								8.3				
47	*Enterobacter cloacae*	1.81	91.18	140.8							6.0	6.5	7.4	7.9	8.8
48	*Pseudomonas aeruginosa*										8.2				
49	*Pseudomonas aeruginosa*														
50	*Enterobacter cloacae*	3.38	140.8								7.6				
51	*Enterobacter cloacae*	140.8									7.6				
52	*Enterobacter cloacae*	163.2									7.6				
53	*Enterobacter cloacae*	3.97	4.76	5.52	11.86	34.33	88.75				7.6				
54	*Enterobacter cloacae*	3.55	4.7	6.44	12.15	87.55					7.6				
55	*Pseudomonas aeruginosa*										7.8				
56	*Pseudomonas aeruginosa*										8.2				
57	*Morganella morganii*										5.4	6.8			
58	*Morganella morganii*										6.8				
59	*Pseudomonas aeruginosa*														
60	*Pseudomonas aeruginosa*														
61	*Enterobacter cloacae*										6.8	7.5			
62	*Enterobacter cloacae*										6.8	7.5			
63	*Pseudomonas aeruginosa*										7.5				
64	*Enterobacter cloacae*										7.6				
65	*Enterobacter cloacae*										7.6				
66	*Enterobacter cloacae*										4.9	7.9			
67	*Pseudomonas* species														
68	*Pseudomonas* species														
69	*Pseudomonas* species										7.6				
70	*Pseudomonas* species														
71	*Pseudomonas aeruginosa*														

Appendix V : Isolate, Plasmid and B-Lactamase Matrix

Isolate		Plasmids (kb)									B - Lactamases (pI)				
No.	Name	1	2	3	4	5	6	7	8	9	1	2	3	4	5
72	*Pseudomonas aeruginosa*														
73	*Pseudomonas aeruginosa*														
75	*Hafnia alvei*	42.08	119								7.4	7.8			
76	*Escherichia coli*	73.26	92.05								5.4	7.4			
77	*Enterobacter cloacae*	4.15	12.71	180.6							7.6				
78	*Enterobacter cloacae*	4.15	12.71	180.6							7.6	8.0			
79	*Enterobacter cloacae*	4.15	12.71	180.6							7.6	8.0			
80	*Enterobacter cloacae*	4.15	12.71	180.6							6.8	7.6	8.0		
81	*Enterobacter cloacae*	4.15	12.71	180.6							7.6	8.0			
82	*Acinetobacter calco* var *anitratus*	124.4													
83	*Pseudomonas aeruginosa*														
84	*Pseudomonas aeruginosa*														
85	*Pseudomonas aeruginosa*										8.7				
86	*Acinetobacter calco* var *anitratus*														
87	*Pseudomonas aeruginosa*														
88	*Acinetobacter calco* var *anitratus*										8.5				
89	*Flavobacterium odoratum*										7.8				
90	*Flavobacterium odoratum*														
91	*Pseudomonas aeruginosa*										8.1				
92	*Enterobacter cloacae*	2.32	103	180.6							7.1	7.6	8.1		
93	*Pseudomonas aeruginosa*										8.1				
94	*Enterobacter cloacae*	2.32	103	180.6							7.1	7.6	8.1		
95	*Pseudomonas fluorescens*										8.3				
96	*Acinetobacter calco* var *anitratus*	81.94													
97	*Citrobacter freundii*	7.48									8.1				
98	*Pseudomonas aeruginosa*														
99	*Stenotrophomonas maltophilia*														
100	*Enterobacter cloacae*	4.41	9.01	161.9							8.1				
101	*Enterobacter cloacae*	4.41	9.01	33.97	161.9						7.7	8.3	8.5		
102	*Enterobacter cloacae*	4.41	9.01	161.9							8.3	8.5			
103	*Pseudomonas aeruginosa*										8.7				
104	*Pseudomonas species*										8.4				
105	*Acinetobacter calco* var *anitratus*	9.83									8.5				

Appendix V : Isolate, Plasmid and B-Lactamase Matrix

Isolate		Plasmids (kb)									B - Lactamases (pI)				
No.	Name	1	2	3	4	5	6	7	8	9	1	2	3	4	5
106	*Acinetobacter calco* var *anitratus*										8.1				
107	*Acinetobacter calco* var *anitratus*	9.83									8.0				
108	*Acinetobacter calco* var *anitratus*										8.0				
109	*Pseudomonas aeruginosa*										7.7				
110	*Pseudomonas aeruginosa*														
111	*Acinetobacter calco* var *anitratus*	16.46	67.94								7.7				
112	*Acinetobacter calco* var *anitratus*	16.46	67.94								7.9				
113	*Morganella morganii*										6.5	7.3	7.9		
114	*Acinetobacter calco* var *anitratus*	16.46	67.94								8.7				
115	*Acinetobacter calco* var *anitratus*	9.83									8.7				
116	*Enterobacter cloacae*	4.51	11.04	214.5							7.9				
117	*Pseudomonas aeruginosa*	6.68	39.75								7.9				
118	*Citrobacter freundii*										8.7				
119	*Citrobacter freundii*										8.5				
120	*Acinetobacter calco* var *anitratus*	9.83									8.9				
121	*Enterobacter species*	165.3									7.9				
122	*Citrobacter freundii*										7.9	8.7			
123	*Acinetobacter calco* var *lwoffii*	102.4									8.7				
124	*Pseudomonas aeruginosa*														
125	*Pseudomonas aeruginosa*										8.3				
126	*Enterobacter cloacae*	4.17	13.43	120							6.1	6.5	6.9	7.8	
127	*Acinetobacter calco* var *anitratus*										7.8				
128	*Pseudomonas* species														
129	*Pseudomonas aeruginosa*										8.0				
130	*Acinetobacter calco* var *anitratus*										8.9				
132	*Acinetobacter calco* var *anitratus*										7.9				
133	*Klebsiella pneum pneumoniae*	4.93	10.25								5.4	7.6			
134	*Enterobacter cloacae*	165.3									7.7				
135	*Enterobacter species*	197.5									7.8				
137	*Acinetobacter calco* var *anitratus*	5.05	5.85	50.87							7.9	8.6			
138	*Citrobacter freundii*	5.39	6.66	17.84	26.8	54.3	85.28	105			8.4				
139	*Pseudomonas aeruginosa*														
140	*Klebsiella pneum pneumoniae*	5.05	47.97	168.2							5.3	7.6			

Appendix V : Isolate, Plasmid and B-Lactamase Matrix

Isolate		Plasmids (kb)									B - Lactamases (pI)				
No.	Name	1	2	3	4	5	6	7	8	9	1	2	3	4	5
141	*Enterobacter cloacae*	168.2									8.0				
142	*Enterobacter cloacae*	197.5									8.0				
143	*Acinetobacter calco* var *anitratus*	32.17	96.36								8.0				
144	*Pseudomonas aeruginosa*										8.6				
145	*Pseudomonas aeruginosa*										8.3				
146	*Enterobacter cloacae*	120									8.0				
147	*Enterobacter cloacae*	120									8.0				
148	*Enterobacter cloacae*	113.2									8.0				
149	*Enterobacter cloacae*	5.89	22.41	45.71	171.2						8.6				
150	*Enterobacter cloacae*	5.89	22.41	45.71							8.6				
151	*Pseudomonas aeruginosa*										8.4				
152	*Pseudomonas species*														
153	*Citrobacter freundii*										8.6				
154	*Acinetobacter calco* var *anitratus*	11.91													
157	*Morganella morganii*										6.8				
158	*Pseudomonas species*										8.4				
160	*Pseudomonas aeruginosa*										7.9	8.4			
161	*Enterobacter cloacae*										8.0				
162	*Pseudomonas aeruginosa*										8.4				
163	*Pseudomonas aeruginosa*										7.4	7.9			
165	*Citrobacter freundii*										8.4				
166	*Pseudomonas aeruginosa*														
167	*Hafnia alvei*	4.7	5.8	10.71	14.34	50.83	58.69				7.8				
168	*Hafnia alvei*	4.7	5.8	10.71	14.34						7.8				
169	*Hafnia alvei*	4.7	5.8	10.71	14.34	50.83					7.8				
170	*Pseudomonas aeruginosa*														
171	*Pseudomonas aeruginosa*										8.5				
172	*Pseudomonas aeruginosa*														
173	*Acinetobacter calco* var *anitratus*										8.5				
174	*Enterobacter cloacae*	2.32	3.87								6.8	8.6			
177	*Pseudomonas aeruginosa*										7.4	7.9			
178	*Enterobacter cloacae*	2.32	3.87								8.6				
182	*Pseudomonas aeruginosa*										7.7				

Appendix V : Isolate, Plasmid and B-Lactamase Matrix

Isolate		Plasmids (kb)									B - Lactamases (pI)				
No.	Name	1	2	3	4	5	6	7	8	9	1	2	3	4	5
183	*Pseudomonas aeruginosa*										7.4	7.9			
184	*Enterobacter cloacae*	2.15	3.3	3.72	4.21						6.9	8.7			
190	*Pseudomonas aeruginosa*														
191	*Acinetobacter calco* var *anitratus*										5.6	8.8			
195	*Acinetobacter calco* var *anitratus*										8.9				
196	*Acinetobacter calco* var *anitratus*	11.25									7.7				
197	*Enterobacter cloacae*										8.6				
198	*Pseudomonas aeruginosa*														
199	*Enterobacter cloacae*										8.2				
200	*Escherichia coli*	4.93	11.25	55.45							8.5				